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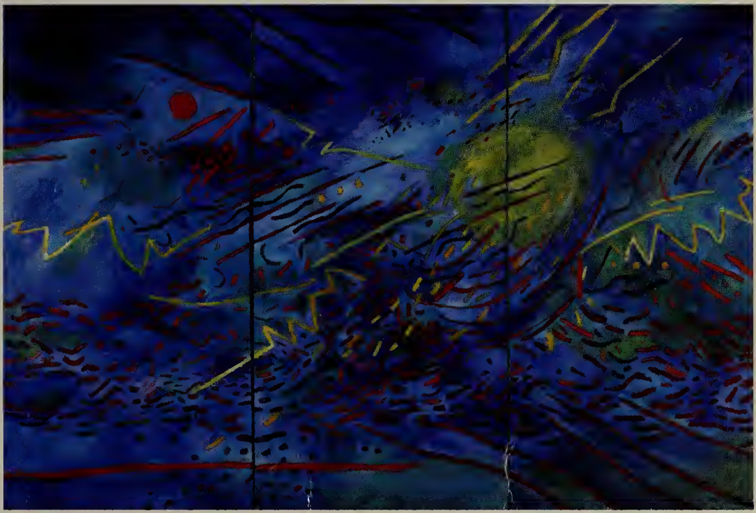

Ethanol and Intracellular Signaling: From Molecules to Behavior

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ETHANOL AND INTRACELLULAR SIGNALING: FROM MOLECULES TO BEHAVIOR

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Key words are included in the beginning of each article. These descriptors are drawn from *The Alcohol and Other Drug Thesaurus: A Guide to Concepts and Terminology in Substance Abuse and Addiction, Second Edition, 1995* and may be used to retrieve this monograph in the Alcohol and Alcohol Problems Science Database (commonly referred to as ETOH).

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FOREWORD

Over the last quarter century, a number of significant advances have been made toward understanding the mechanisms underlying alcohol's actions in the brain and how these actions, in turn, influence the development of specific alcohol-related behaviors. A picture has begun to emerge of intricate, detailed pathways or circuits of communication involved in the development of alcoholism. Thus, understanding how alcohol affects cellular communication to produce alcohol-related behaviors, such as craving, tolerance, withdrawal, and impaired control, is an important step toward developing therapeutic interventions to improve treatment outcome.

To help stimulate research interest in and attention to this important area, the National Institute on Alcohol Abuse and Alcoholism sponsored a meeting as a satellite to the 1998 Meeting of the International Society for Biomedical Research on Alcoholism to bring together investigators working in different cellular and functional contexts and to integrate studies of the underlying cellular and mechanistic aspects of alcohol dependence with studies of the responses of the organism as a whole. This monograph, *Ethanol and Intracellular Signaling: From Molecules to Behavior,* is derived from papers presented at that meeting. The papers presented herein provide a unique perspective on the importance of looking at the whole animal as well as its biological parts.

I wish to thank the many individuals who participated in this meeting and to extend my appreciation to the scientists whose excellent presentations form the basis of this monograph. I am particularly grateful to Drs. Jan B. Hoek, Adrienne S. Gordon, Daria Mochly-Rosen, and Sam Zakhari for their hard work, both in organizing the satellite meeting and in preparing this monograph. Their efforts in making possible the sharing of good science with a broad audience are greatly appreciated.

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PREFACE

Alcohol is a ubiquitous molecule that affects cells both directly and by interacting with various intracellular signaling cascades. Broadly speaking, signaling may be defined as a cell's internal biochemical response to the activation by external chemical messengers of receptor proteins in the cell membrane. Although scientists have known that alcohol affects signaling, researchers first focused on trying to identify specific receptors that may be targeted by alcohol. However, unlike most other abused drugs, alcohol does not have a specific neurotransmitter binding site in the brain. By the late 1980s, research had established that intoxicating concentrations of alcohol can alter the function of ion channels activated by specific neurotransmitters. This finding triggered a spate of publications on alcohol's effects on specific ion channels.

However, the complex behaviors associated with alcohol use cannot be attributed solely to a limited number of specific chemical interactions. This realization has led to a renewed emphasis on alcohol's effects on intersecting pathways of neuronal communication that coordinate the activities of multiple brain regions. Alcohol's effects on these pathways can help account for relatively short-term features of alcoholism, such as acute tolerance, physical withdrawal symptoms, the initiation of reinforcement, and the establishment of dependence. Transient disruption of the brain's communications network does not account for longer term manifestations of alcoholism—for example, uncontrolled craving that may contribute to relapse years after cessation of drinking.

An understanding of intracellular signaling not only helps explain some of alcohol's acute effects but also provides the link between a neuron's initial response to alcohol and persistent alterations in neuronal function. These alterations, perhaps analogous to the neural plasticity involved in memory, may involve long-term changes in both cell metabolism and gene expression. By affecting permanent changes in the structure and function of receptors, ion channels, and synaptic connections within neuronal pathways, signaling closes the loop between extracellular neurotransmitter-receptor interactions and the potentiation of systems-level processes that underlie alcohol-related behavior.

Intracellular signaling comprises a complex network of mutually interacting processes. Among the common themes that emerge is the regulatory role of protein-phosphorylating enzymes (e.g., protein kinases). By attaching a phosphate group to the target molecule, these enzymes can activate other regulatory enzymes (e.g., G proteins), modify the function of ion channels, or activate transcription factors that initiate the expression of specific genes.

This monograph explores three major areas of relevant research: the function of calcium as a second messenger, the regulatory actions of the family of enzymes known as protein kinases, and the role of gene expression in mediating long-term neuronal changes induced by alcohol.

CALCIUM SIGNALING MECHANISMS

Many signaling pathways involve mobile molecules called second messengers. These molecules may regulate short-term events (e.g., ion channel activity and neurotransmitter release) as well as longer term processes (e.g., synaptic plasticity, memory, and learning). Chapter 1 discusses perturbations of calcium-mediated signaling pathways in alcohol-fed animals. Chapter 2 provides evidence that altered regulation of calcium-specific ion channels by G proteins may influence shifts in alcohol sensitivity following chronic alcohol exposure.

Chronic alcohol consumption also impairs the function of intracellular receptors for the second messenger inositol triphosphate (IP3), which helps regulate the release of calcium from intracellular storage sites. As noted in chapter 3, alcohol-induced dysregulation of this process may promote the accumulation of intracellular proteins associated with alcohol-induced liver injury.

PROTEIN KINASE SIGNALING

The phosphorylating activity of protein kinases is relatively nonspecific. Consequently, these enzymes must be transported to the appropriate substrate and attached to nearby anchoring proteins. Administration of alcohol to neurons grown in culture (discussed in chapter 4) results in dramatic shifts in protein kinase localization, potentially resulting in the activation of inappropriate regulatory proteins. Chapter 5 presents evidence suggesting that translocation and activation of specific types of protein kinase are involved in one of the major intracellular signal transduction pathways that underlie the consistent association of low risk for coronary heart disease with moderate alcohol consumption.

Some of alcohol's long-term adverse health effects involve inhibition of cell proliferation and development pathways. Studies described in chapters 6 and 7 demonstrate that alcohol inhibits the activation by protein kinases of growth pathways that involve insulin and an insulin-like growth factor. This mechanism may contribute to the impaired regeneration of alcohol-damaged liver cells observed in cirrhosis as well as the neuronal death associated with fetal alcohol syndrome. Chapter 8 suggests that alcohol-induced protein kinase inhibition inhibits the production of anti-inflammatory factors, potentially increasing susceptibility to infectious diseases.

GENES AND BEHAVIOR

Complex alcohol-related behavior results from the interaction of multiple genes, each of which reflects a relatively small part of trait variability. Chapter 9 explains how genetic mapping strategies can help pinpoint the locations of quantitative trait loci on chromosomes. This chapter also reviews genetic engineering approaches that include observing the behavior of mice that have undergone deletion of specific genes (i.e., knockout mice). For example, later chapters in the monograph describe alcohol's effects on strains of knockout mice that lack genes for, respectively, a protein kinase (chapter 14) and a neurotransmitter (chapter 15). Chapter 13 describes experiments using naturally occurring fruit fly mutants that exhibit abnormal responses to alcohol.

Chapters 10 through 12 focus on structural changes in neural pathways resulting from alcohol's effects on gene expression. Of particular interest is the identification of a unique set of genes in the prefrontal cortex that appears to be involved in synaptic remodeling. According to studies reported in chapter 10, these genes are expressed only in animals that have developed neuroadaptive changes to long-term alcohol exposure. Additional studies implicate a specific protein kinase in alcohol-induced neuroadaptation through pathways involving the expression of transcription factors (chapter 11) and the regulation of calcium channels (chapter 12).

The contributors to this monograph are at the forefront of their disciplines and have devoted immense time and effort to preparing their chapters. Without their dedicated efforts, this monograph would not have been possible. We also would like to acknowledge the dedicated help of both Diane Miller and Diana O'Donovan at the National Institute on Alcohol Abuse and Alcoholism and Dianne Welsh and her staff—Patricia Freedman, John Doria, and Elaine Garber—at CSR, Incorporated. Their remarkable attention to detail and pursuit of excellence are greatly appreciated.

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ABBREVIATIONS AND ACRONYMS

AC	adenylate cyclase
ACTH	adrenocorticotropic hormone
ADP	adenosine diphosphate
AHMD	alcoholic heart muscle disease
AKAP	A kinase anchoring protein
ALLN	*N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal
Ang II	angiotensin II
AP-1	activator protein 1
ApoB-100	apolipoprotein B-100
BDNF	brain-derived neurotrophic factor
β-PMA	β-phorbol 12-myristate 13-acetate
CaM	calmodulin
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CHD	coronary heart disease
cm	centimeter(s)
CNS	central nervous system
cpm	counts per minute
CREB	cAMP-response element binding protein
CYP2E1	cytochrome P-450_{2E1}
DAG	diacylglycerol
DADLE	D-ala-D-leu enkephalinamide
dbcAMP	dibutyryl cyclic adenosine monophosphate
dCTP	deoxycytidine triphosphate
DHP	dihydropyridine
dL	deciliter(s)
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EC_{50}	median effective concentration
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGTA	ethyleneglycoltetraacetic acid
EMSA	electromobility shift assays
EPSP	excitatory postsynaptic potential
ER	endoplasmic reticulum
EtRG	ethanol-responsive gene
FBS	fetal bovine serum
g	grams(s) *or* gravity
GABA	gamma-aminobutyric acid

GABA_A	gamma-aminobutyric acid type A
GDP	guanosine diphosphate
GDP-β-S	guanosine-5′-O-(2-thiodiphosphate)
GHRH	growth hormone–releasing hormone
GRF	growth hormone–releasing factor
GTP	guanosine triphosphate
GTP-γ-S	guanosine-5′-O-(3-thiotriphosphate)
HGF	hepatocyte growth factor
IGF-I	insulin-like growth factor I
IGF-IR	insulin-like growth factor I receptor
IL-1β	interleukin-1β
IP_3	inositol trisphosphate *or* inositol 1,4,5-trisphosphate
IP_3R	inositol trisphosphate receptor
IRS-1	insulin receptor substrate 1
IU	International Unit(s)
kb	kilobase
kd	kilodalton(s)
kg	kilogram(s)
KN-62	1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine
LORR	loss of righting reflex
LPS	lipopolysaccharide
LTP	long-term potentiation
LV	left ventricular
M	molar
MAP	mitogen-activated protein [kinase]
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant peptide-1
MET	mean elution time
mg	milligram(s)
MHC	major histocompatibility complex
mL	milliliter(s)
mm	millimeter(s)
mM	millimolar *or* millimole(s)
mOsmol	milliosmole(s)
mRNA	messenger ribonucleic acid
ms	millisecond(s)
µg	microgram(s)
µL	microliter(s)
µM	micromolar *or* micromole(s)
µm	micrometer(s)
mV	millivolt(s)

nA	nanoampere(s)
NaD^+	oxidized form of nicotinamide adenine dinucleotide
NEM	*N*-methylmaleimide
NF-κB	nuclear factor-κB *or* nuclear regulatory factor κB
ng	nanogram(s)
NGF	nerve growth factor
NIAAA	National Institute on Alcohol Abuse and Alcoholism
nm	nanometer(s)
nM	nanomolar *or* nanomole(s)
NMDA	*N*-methyl-D-aspartate
NMDAR	*N*-methyl-D-aspartate receptor
ODN	oligodeoxynucleotide
oz	ounce(s)
pA	picoampere(s)
PACAP	pituitary adenylyl cyclase activating peptide
PCNA	proliferating cell nuclear antigen
PDGF	platelet-derived growth factor
pg	picogram(s)
PGPH	peptidylglutamyl peptide hydrolyzing [activity]
PH	pleckstrin homology [domain]
PI3K	phosphatidylinositol 3-kinase
P-IκBα	phospho-IκBα
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	cAMP-dependent protein kinase *or* protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PLC	phospholipase C
pM	picomolar
PMA	phorbol 12-myristate 13-acetate *or* 4β-phorbol 12-myristate 13-acetate
PMSF	phenylmethyl sulfonyl fluoride
PNET	primitive neuroectodermal tumor [cell]
PNET2	primitive neuroectodermal tumor type 2
POMC	proopiomelanocortin
PSD	postsynaptic density
PTX	pertussis toxin
PY-IRS-1	tyrosyl phosphorylated insulin receptor substrate 1
QTL	quantitative trait locus
RACK	receptor for activated C-kinase
RICK	receptor for inactive C-kinase
RNA	ribonucleic acid
ROS	reactive oxygen species

RPMI	Roswell Park Memorial Institute
SD	standard deviation
SDS	sodium dodecyl sulfate
SE	standard error
SEB	staphylococcal enterotoxin B
SEM	standard error of the mean
SRE	serum responsive element
TBS	Tris-buffered saline
TNF	tumor necrosis factor
TNFα	tumor necrosis factor alpha
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TUNEL	terminal transferase [14-biotin] dUTP end-labeling
$V_{1/2}$	half-maximal inactivation potential
V_h	holding potential
vol %	volume percent
vol/vol	volume per volume
WSP	withdrawal seizure-prone

ETHANOL AND Ca^{2+} SIGNALING PATHWAYS

Chapter 1

Ethanol and Intracellular Ca^{2+} Signaling Pathways

Jan B. Hoek, Ph.D., and Andrew P. Thomas, Ph.D.

Key words: cell signaling; intracellular transport; calcium; homeostasis; ethanol; phospholipases; chronic AODE (effects of AOD [alcohol or other drug] use, abuse, and dependence); receptor proteins; binding proteins; cell electrophysiology; biological inhibition; biological activation; cell growth and differentiation; laboratory study

Intracellular signal transduction processes have emerged as a major target for ethanol in a variety of cells and tissues. Ethanol acts on G protein–coupled signaling systems (Hoffman and Tabakoff 1990; Hoek et al. 1992), tyrosine kinase–dependent signaling pathways (Resnicoff et al. 1993; Saso et al. 1997; Chen et al. 1999; de la Monte et al. 1999), ion channels (Wang et al. 1994; Peoples et al. 1996; Mihic et al. 1997; Chandler et al. 1998; Chu et al. 1998; see also chapter 2 in this monograph), and intracellular protein kinases and phosphatases (Higashi et al. 1996; Diamond and Gordon 1997; Miyamae et al. 1998). In some instances, ethanol affects the basal activity of the signaling processes, generating an ethanol-induced signal that may be superimposed on other signals or that may initiate a desensitization to subsequent receptor-mediated signals. More commonly, ethanol affects the response to receptor activation by hormones, cytokines, or other agonists, by direct or indirect mechanisms. Evidence has emerged recently that intracellular signaling processes often depend on localization signals that generate a spatially restricted response. Interesting new evidence has shown that ethanol may alter the efficacy of

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such localization signals, or even alter their targeted locus (Gordon et al. 1998; Constantinescu et al. 1999). The associated changes in the intracellular regulatory environment may contribute to pleiotropic ethanol-induced changes in the cell, ranging from metabolism to gene transcriptional control (e.g., see Ding et al. 1996).

These effects of ethanol do not always require its metabolism but often appear to be mediated by its hydrophobicity, for example, by targeting specific lipid domains or by binding to hydrophobic sites on proteins and thereby interfering with protein-protein or lipid-protein interactions (Franks and Lieb 1987; Channareddy et al. 1996; Peoples et al. 1996). These properties allow ethanol effects to be observed in a wide variety of cells and tissues, irrespective of the presence of ethanol-metabolizing enzymes. However, in tissues such as the liver, where ethanol is actively metabolized, additional avenues are available for ethanol to affect signaling processes that are associated with redox stress, acetaldehyde formation, or the generation of reactive oxygen or nitrogen species, which may be mediated through stress-activated protein kinases or nuclear factor-κB (NF-κB).

Our studies have focused on the actions of ethanol on phospholipase C (PLC)–mediated Ca^{2+} signaling in the liver. Polyphosphoinositide-specific PLC constitutes one of the predominant effector systems for different receptors. At least 16 different isozymes of polyphosphoinositide-specific PLC are known and are divided into three main classes—β, γ, and δ. These isozymes are activated by distinct mechanisms, depending on the nature of the receptor system involved (Rhee and Bae 1997). In liver, PLCβ1 is the predominant isoform that is linked to heptahelical G protein–coupled receptors, such as the vasopressin (V_{1a}) or angiotensin II receptors. PLCβ1 is activated by the α subunit of heterotrimeric G proteins of the G_q/G_{11} class (Blank et al. 1991), a subfamily of G proteins that is not subject to ADP-ribosylation by pertussis toxin or cholera toxin. The mechanism of activation of PLCβ1 by G_q/G_{11} follows the standard model of G protein activation, with the receptor acting to promote the exchange of guanosine disphosphate (GDP) for guanosine triphosphate (GTP) on the α subunit of the trimeric G protein complex, followed by the dissociation of the α and βγ subunits and the activation of the effector enzyme by the GTP-bound form of the α subunit. The α subunit returns to the inactive state following hydrolysis of protein-bound GTP by its intrinsic GTPase activity; in the case of the $G\alpha_q/G\alpha_{11}$ subunits, this intrinsic GTPase activity is very low, but GTP hydrolysis is markedly enhanced by the interaction with PLCβ1 (Berstein et al. 1992). In addition, the GTPase activity is controlled by proteins that enhance their intrinsic GTPase activity (RGS proteins) (Berman and Gilman 1998). G protein βγ subunits are also capable of activating effector enzymes, including PLCβ2 (Park et al. 1993).

Other PLC isoforms respond to different signaling mechanisms. PLCγ isoforms are activated through tyrosine kinase receptors, including the epidermal growth factor (EGF) receptor and the hepatocyte growth factor (HGF) receptor. The enzyme binds to the autophosphorylated receptor through its SH2 domains and tyrosine phosphorylation of the enzyme promotes its interaction with its substrate, phosphatidylinositol 4,5-bisphosphate (PIP_2) by mechanisms that have not been adequately resolved (Wahl et al. 1992). PLCδ isoforms bind to membrane domains enriched in specific phospholipid species, in part through their specific pleckstrin homology (PH) domains, and they are activated in response to elevation of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). Hence, PLCδ may be activated secondarily following $[Ca^{2+}]_i$ elevation caused by other signals (Kim et al. 1999). In addition, there is evidence that α_{1B} adrenergic receptors activate PLCδ1 through Gh, a different class of G protein that also acts as a transglutaminase which is involved in protein cross-linking (Feng et al. 1996; Im et al. 1997). These two different enzymatic activities of the Gh protein appear to have an inverse relationship, although the relevant molecular mechanisms remain to be elucidated. Ethanol activates tissue transglutaminase activity (Sessa et al. 1997) and inhibits PLCδ1 activation and Ca^{2+} mobilization through this pathway (Wu et al. in press).

All phosphoinositide-specific PLC isoforms ultimately are thought to

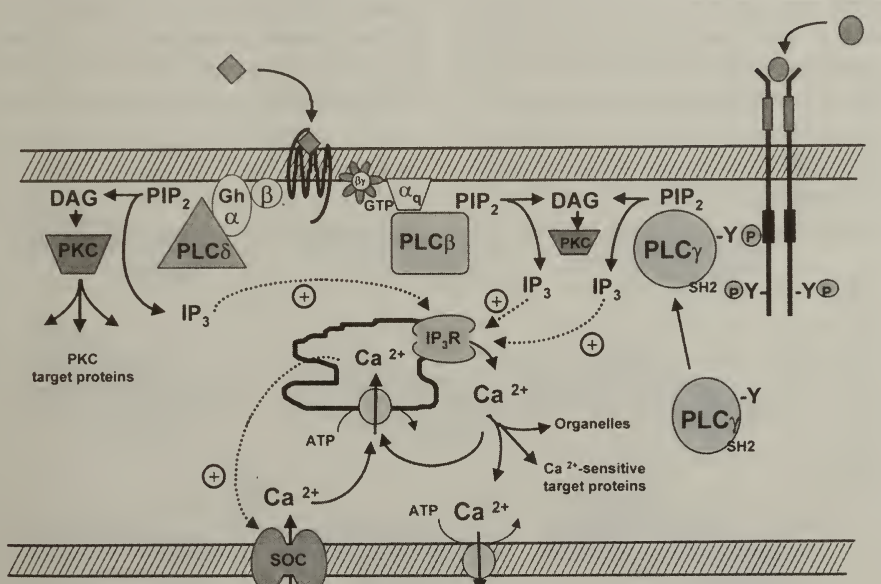

Figure 1. Scheme of phospholipase C (PLC) activation and changes in Ca^{2+} homeostasis activated through G protein–coupled receptors. ATP = adenosine triphosphate; DAG = diacylglycerol; GTP = guanosine triphosphate; IP_3 = inositol 1,4,5-trisphosphate; IP_3R = inositol 1,4,5-trisphosphate receptor; P = phosphate; PIP_2 = phosphatidylinositol 4,5-bisphosphate; PKC = protein kinase C; SOC = store-operated Ca^{2+} channels; Y = tyrosine.

activate the same reaction, namely the hydrolysis of PIP_2 to generate the second messenger molecules diacylglycerol (DAG), which activates protein kinase C (PKC), and inositol 1,4,5-trisphosphate (IP_3), which causes release of Ca^{2+} from intracellular reservoirs through the IP_3 receptor (IP_3R), an IP_3-gated Ca^{2+} channel located in the endoplasmic reticulum (ER) (see figure 1). Much has been learned in the past 15 years concerning the mechanisms that regulate $[Ca^{2+}]_i$ in the context of hormonal stimulation and how these processes interact with Ca^{2+} concentration changes in various organelles. There is evidence that ethanol affects the Ca^{2+} homeostatic mechanisms at a number of different sites, and recent evidence suggests that in the liver these changes may have physiological relevance for a variety of Ca^{2+}-dependent processes. In this chapter, we will discuss these recent insights into Ca^{2+} homeostasis and the interactions of ethanol with these processes and consider evidence of its mechanism of action.

CELLULAR Ca^{2+} HOMEOSTASIS AND THE INCREASE OF INTRACELLULAR Ca^{2+} BY IP_3

The IP_3R is a ligand-gated high conductance Ca^{2+} channel located in the ER membrane (see Patel et al. 1999*a* for a review). Binding of IP_3 to its receptor causes opening of the Ca^{2+} channel, resulting in the rapid release of ER Ca^{2+} stores with consequent elevation of $[Ca^{2+}]_i$ from their normal resting levels of around 100 nM to peak concentrations that can reach the µM range in specific locations in the cell. Characteristically, in cells such as

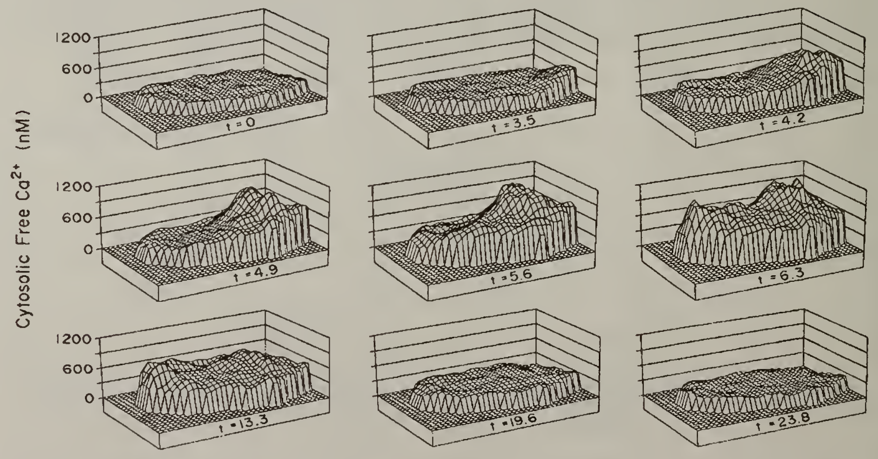

Figure 2. Oscillatory Ca^{2+} waves induced by phenylephrine in a single rat hepatocyte. The images map the changes in free Ca^{2+} concentration across a single fura-2–loaded hepatocyte, as determined from the 340/380 nm fluorescence ratio at different times (numbers in seconds) following stimulation with 10 µM phenylephrine. Adapted from Rooney and Thomas 1993.

liver parenchymal cells that generate an IP_3-mediated Ca^{2+} response upon hormonal stimulation, the cytosolic Ca^{2+} elevation is not sustained but oscillates with a frequency that is dependent on the strength of the hormonal signal. The Ca^{2+} transients appear to originate in subregions of the cell and spread from there as a wave of Ca^{2+} elevation throughout the cell, as illustrated in figure 2.

The factors controlling these characteristic Ca^{2+} response patterns have been the subject of intense scrutiny in the past decade, but remain only partly understood. The IP_3R itself is an important site where this control is determined (Rooney and Thomas 1993). IP_3 binding and IP_3-induced Ca^{2+} release through the IP_3R is regulated by Ca^{2+} concentrations both in the cytosol and in the lumen of the ER, and multiple Ca^{2+} binding sites are present with Ca^{2+} binding characteristics that vary between different IP_3R isoforms. In liver cells, IP_3 binding to the receptor is enhanced severalfold by $[Ca^{2+}]$ in the range of 0.1–1 µM, but is inhibited at higher $[Ca^{2+}]$. It is thought that these properties contribute to an autocatalytic stimulation of Ca^{2+}-dependent Ca^{2+}-release from the ER stimulated by a local increase in IP_3. Higher Ca^{2+} concentrations result in shutting off the receptor and allowing the reuptake of Ca^{2+} into the ER through the ER Ca^{2+} pump (Rooney and Thomas 1993). Figure 3 shows a schematic representation of how this pattern of Ca^{2+} release and reuptake can generate an oscillating wave pattern in the cell.

Other Ca^{2+} transport systems are integrated with the IP_3-induced Ca^{2+} release patterns. The elevated cytosolic $[Ca^{2+}]$ activates Ca^{2+} efflux through the Ca^{2+} pump in the plasma membrane. At the same time, a Ca^{2+} influx pathway is activated ("capacitative Ca^{2+} entry"; see Thomas et al. 1996), mediated by a plasma membrane Ca^{2+} channel that is opened by the release of Ca^{2+} from the ER (store-operated Ca^{2+} channels, SOC). The combined

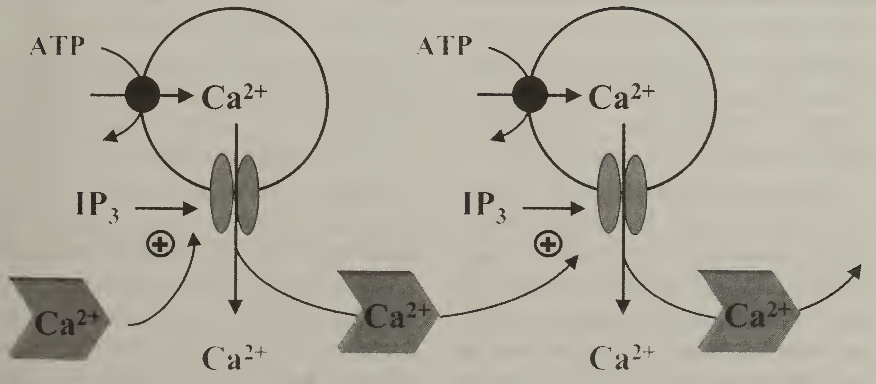

Figure 3. Schematic model of the generation of inositol 1,4,5-trisphosphate (IP_3)–induced Ca^{2+} waves. ATP = adenosine triphosphate. Adapted from Rooney and Thomas 1993.

activity of these transport processes must be carefully regulated to enable the cell to retain its total Ca content despite marked fluctuations in the intracellular distribution of this cation. Intracellular organelles also participate in the Ca^{2+} transients generated by IP_3. Nuclear [Ca^{2+}] follows the changes in cytosolic [Ca^{2+}], probably enhanced by IP_3 receptors acting on Ca^{2+} storage sites in the nuclear envelope (Lin et al. 1994). Mitochondria rapidly accumulate Ca^{2+} released from the ER and show matrix Ca^{2+} oscillations that coincide with the changes in $[Ca^{2+}]_i$ (Hajnoczky et al. 1995). Mitochondrial Ca^{2+} uptake is probably enhanced by the rapid local release of ER Ca^{2+} through the IP_3R and responds to locally elevated [Ca^{2+}] in the immediate vicinity of the IP_3 release channel (Rizzuto et al. 1993, 1998). The resulting changes in matrix free [Ca^{2+}] regulate intramitochondrial oxidative metabolism (Robb-Gaspers et al. 1998). In addition, the mitochondrial release of Ca^{2+} may contribute to the pattern of Ca^{2+} waves across the cell by affecting the local regulation of IP_3R function (Jouaville et al. 1998; Landolfi et al. 1998). Hence, Ca^{2+} waves function to integrate hormonal signals throughout the cell and its compartments.

The coordination of Ca^{2+} waves also operates at the level of the whole tissue. Confocal microscopic studies on Ca^{2+} response patterns in the perfused liver demonstrate that the oscillatory responses are preserved in the intact tissue and may, in fact, operate to integrate functional responses across the liver acinus (Robb-Gaspers and Thomas 1995). Characteristically, when the liver was perfused with a low concentration (80 pM) of vasopressin or other Ca^{2+} mobilizing hormone, a transient Ca^{2+} elevation was initiated in a restricted number of individual hepatocytes, mostly in the periportal region of the liver, from where it spread through gap junctions to other cells across the liver acinus. Thus, this oscillatory Ca^{2+} wave pattern can provide a device to integrate the tissue's functional responses activated by hormonal stimulation.

ETHANOL AND Ca^{2+} SIGNALING IN THE LIVER

Ethanol treatment of isolated hepatocytes (or other cell types) affects PLC-mediated signaling responses at different levels. Acute ethanol exposure suppresses the response to stimulation with a variety of hormones that are coupled to PLC (Higashi and Hoek 1991). At least part of this suppressive effect is located at the level of PLC activation, since the rate of IP_3 formation induced by vasopressin or other hormones is inhibited. Although ethanol addition itself, in the absence of other hormonal stimuli, triggers a transient PLC activation in hepatocytes (Hoek et al. 1987), it is not clear that these two phenomena are related. Specifically, when the cellular Ca^{2+} responses were compared at the level of individual cells, it appeared that there was substantial variation between cells in the same population (Hoek et al. 1993). Some cells had no detectable Ca^{2+} transient when stimulated with ethanol itself, but ethanol treatment still affected their response to a subsequent addition of vasopressin or phenylephrine. By contrast, hormonal responses in other cells were entirely unaffected by ethanol, even though the cells did show a marked Ca^{2+} transient when treated with ethanol itself. Interestingly, ethanol suppression of PLC-mediated responses have been reported in several cell lines (Alling et al. 1993, Larsson et al. 1998, Bokkala et al. 1999), but these have generally not shown evidence of ethanol directly activating PLC. Hence, the factors responsible for the suppressive effect of ethanol are probably distinct from those that bring about PLC activation by ethanol itself.

Mechanistic studies were done on PLC activation in platelets and in turkey erythrocyte membranes (Rooney et al. 1989). These studies suggest that the receptor–G protein interaction may be a primary target of ethanol that accounts for its stimulatory action. However, the mechanism responsible for its inhibitory effect on hormone-stimulated PLC activity is less well defined. We have provided evidence that PKC plays a role in the inhibition by ethanol of vasopressin-induced Ca^{2+} mobilization in isolated hepatocytes (Higashi and Hoek 1991). PKC is known to inhibit PLCβ1 activation through G protein–coupled receptors. However, the effects of ethanol are additive to those of saturating concentrations of phorbol esters that activate PKC (Higashi and Hoek 1991; Higashi et al. 1996). Hence, ethanol probably acts indirectly—for example, inhibiting a protein phosphatase that dephosphorylates a PKC target protein (Higashi et al. 1994, 1996)—or it may act on PKC through a mechanism that is not mimicked by phorbol esters (Slater et al. 1997).

A different site of action of ethanol was identified by studies of Renard-Rooney and colleagues (1997), who demonstrated that ethanol also acts directly on the IP_3R to inhibit the Ca^{2+} channel opening. Ethanol inhibited both the IP_3-induced Ca^{2+} release in digitonin-permeabilized hepatocytes and, in the same preparation, the IP_3-induced quenching of intraluminal fura-2 by Mn^{2+} entering through the IP_3 receptor, an independent measure of the kinetics of IP_3R opening (Renard-Rooney et al. 1997).

Concentrations of ethanol required for the inhibition of hormonally induced Ca^{2+} responses in isolated hepatocytes and other cells have generally been reported to be in the range of 50–100 mM (Higashi and Hoek 1991; Renard-Rooney et al. 1997; Larsson et al. 1998). However, in liver cells the effective inhibitory concentration depends markedly on the hormone concentration. For instance, in isolated hepatocytes the response to 100 pM vasopressin was significantly suppressed by ethanol concentrations in the range of 10–20 mM (Higashi and Hoek 1991; Higashi et al. 1996), and equivalent concentrations of ethanol inhibited vasopressin-stimulated Ca^{2+} waves in confocal imaging studies on the perfused liver (Gaspers et al. unpublished observations). Since vasopressin concentrations in vivo are likely to be in the picomolar range, concentrations of ethanol that are physiologically readily achievable may

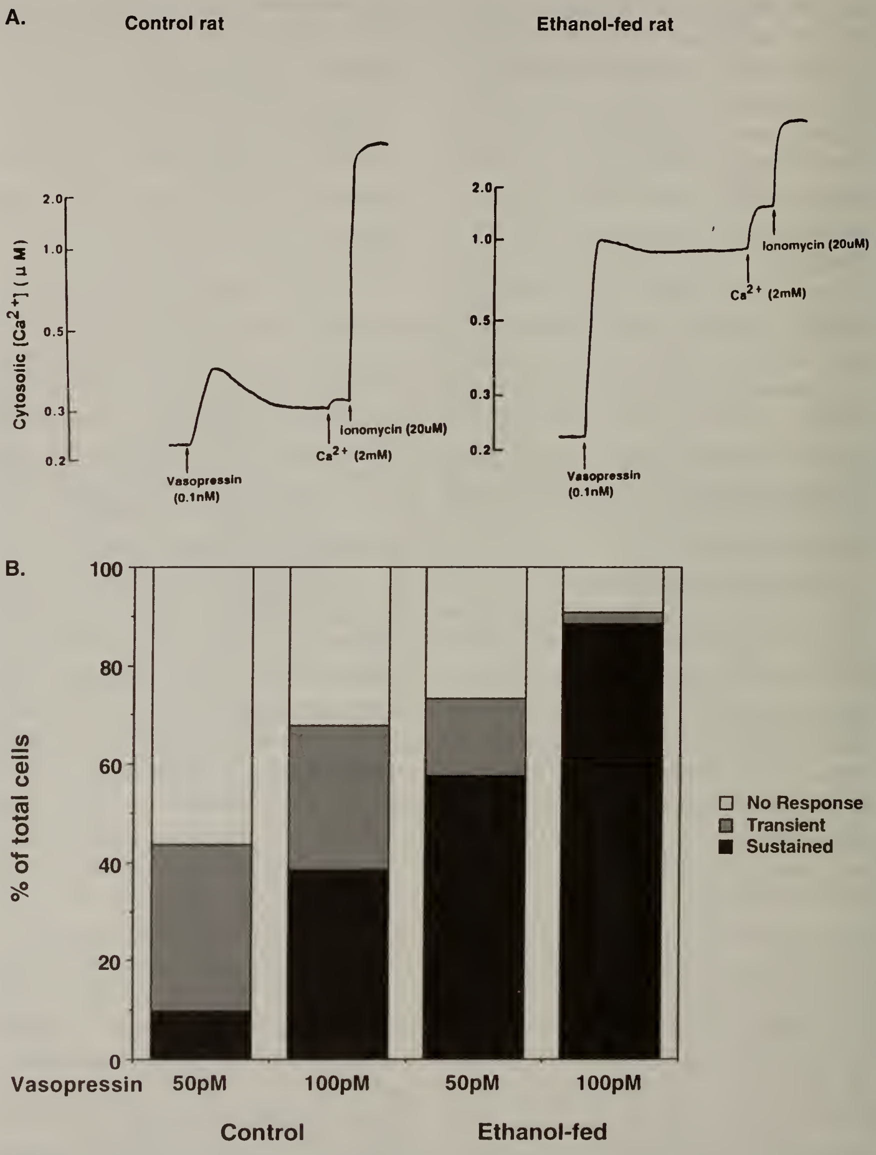

Figure 4. Effects of chronic ethanol treatment on Ca^{2+} response patterns in isolated hepatocytes. (A) Vasopressin-induced changes in $[Ca^{2+}]_i$ in suspensions of indo-1–loaded hepatocytes isolated from ethanol-fed rats and pair-fed control animals. (B) Ca^{2+} response patterns in individual fura-2–loaded hepatocytes from control and chronically ethanol-fed rats analyzed by fluorescence microscopic imaging. The fraction of cells exhibiting transient (single or oscillating) or sustained response patterns was determined in a population of cells visible in a microscope field over a 15-minute period following stimulation with vasopressin (50 pM or 100 pM).

have a significant impact on the response capacity of the liver.

Chronic treatment of animals with ethanol (e.g., following the Lieber-DeCarli protocol [DeCarli and Lieber 1967]) also modifies the hormone-induced Ca^{2+} response capacity of liver cells. Figure 4A illustrates typical changes in Ca^{2+} signals in suspensions of hepatocytes obtained from chronically ethanol-fed rats and their pair-fed controls. At least two important changes were evident in the Ca^{2+} responses in these experiments. First, the Ca^{2+} response to low hormone concentrations was considerably enhanced—that is, the dose-response curve was shifted to the left. Second, the Ca^{2+} elevation was considerably less transient in cells from ethanol-fed animals than in control cells. At the single cell level, these alterations in response pattern were observed as well (figure 4B), with chronic ethanol feeding showing a substantial increase in the fraction of cells that exhibited a Ca^{2+} response to subsaturating hormone levels and more of the responding cells having a sustained elevation of $[Ca^{2+}]_i$ instead of single transients or oscillatory changes in $[Ca^{2+}]_i$. In recent experiments, we found that these changes were also evident in the intact tissue, with a greater sensitivity to low hormone concentrations and more sustained elevations of $[Ca^{2+}]_i$ in the liver from chronically ethanol-fed animals (Gaspers et al. unpublished observations). Moreover, the inhibition by acute ethanol treatment was suppressed, both in the isolated cells and in the tissue. Thus, the alterations in Ca^{2+} response appear to constitute an adaptive change that overcomes in part the inhibitory effects of acute ethanol treatment.

What is the mechanism responsible for the altered Ca^{2+} response following chronic ethanol feeding? Interestingly, the adaptive enhancement of hormonally induced Ca^{2+} response patterns was not evident at the level of the PLC activation: the rate of vasopressin-induced IP_3 formation was not significantly different between cells from control and ethanol-fed animals. Hence, we conclude that the enhanced Ca^{2+} response is predominantly due to changes downstream from the IP_3 formation. Our data suggest that the primary candidate is the IP_3R itself (Nomura et al. 1996). Figure 5 illustrates one of a series of experiments comparing the IP_3R function in control and ethanol-fed animals. In this experiment, digitonin-permeabilized hepatocytes were stimulated with low concentrations of IP_3 to release Ca^{2+} stored in the ER. All cells were pretreated to generate similar Ca^{2+} loading conditions. In cells from control animals, IP_3-induced Ca^{2+} release had an EC_{50} of 100 ± 13 nM, whereas in cells from ethanol-fed animals the EC_{50} was 60 ± 9 nM. IP_3 concentrations required for maximal Ca^{2+} release were not significantly different between the preparations. Hence, these data suggest that the sensitivity of the IP_3R for IP_3 is enhanced.

In other experiments, we found evidence that the enhancement of IP_3 responsiveness was dependent on the prevailing $[Ca^{2+}]_i$. Notably, chronic ethanol treatment appeared to enhance the Ca^{2+} stimulation of IP_3R activation. Thus, we suggest that the adaptive response to chronic ethanol intake affects the autocatalytic process that generates the normal shape of the Ca^{2+} transients and, as a consequence, the cell fails to generate the normal Ca^{2+} response patterns. Figure 6 presents this site of action of ethanol in the context of the model for Ca^{2+} wave progression suggested by Rooney and Thomas (1993).

CONCLUSIONS

A complex set of mechanisms of action emerges by which ethanol affects Ca^{2+} signaling pathways in the liver. In isolated hepatocytes, acute ethanol treatment affects PLC activation mechanisms both directly (at the

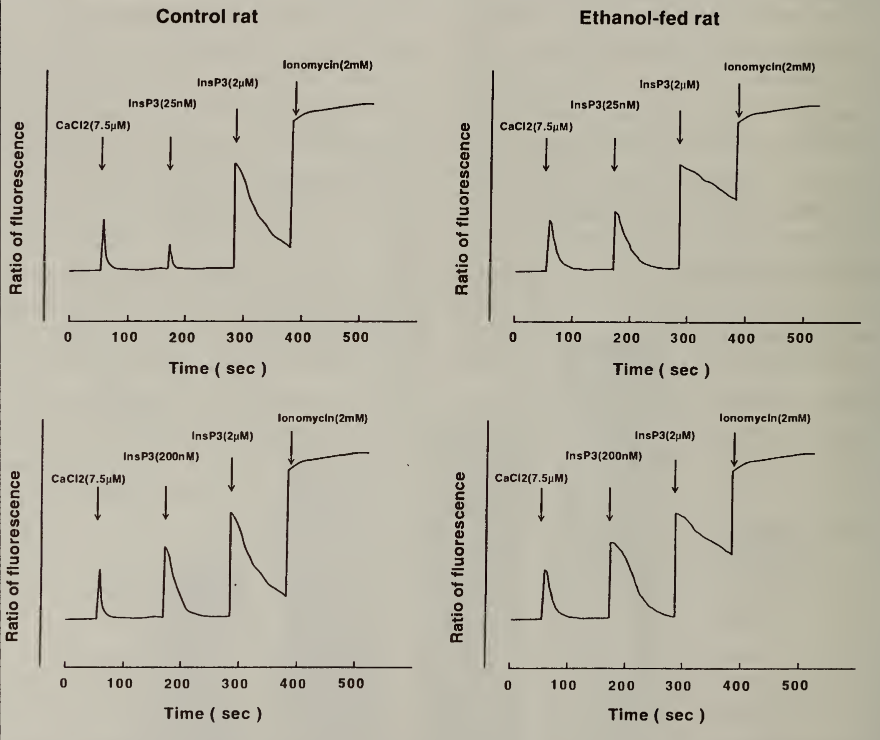

Figure 5. Effect of chronic ethanol treatment on inositol 1,4,5-trisphosphate (InsP3)–induced Ca^{2+} release from the endoplasmic reticulum in digitonin-permeabilized hepatocytes. Hepatocytes from control or ethanol-fed rats were suspended in a nominally Ca^{2+} free Krebs-Henseleit buffer with 2 μM fura-2, permeabilized with digitonin (15 μg/mL), and 1 mM adenosine triphosphate was added to allow Ca^{2+} uptake in the endoplasmic reticular (ER) storage sites. InsP3 was added at different concentrations to induce limited release of Ca^{2+} from the ER storage pools to an initial free Ca^{2+} of 100 nM. At the end of the incubation, a saturating concentration of ionomycin (20 μM) was added to induce maximal Ca^{2+} release. The 340/380 nm fluorescence ratio was determined as an indicator of the free Ca^{2+} concentration in the medium (see Nomura et al. 1996). The traces illustrate a markedly higher Ca^{2+} release at subsaturating concentration of InsP3 in cells from ethanol-fed rats than in control cells for comparable levels of Ca^{2+} loading.

level of receptor–G protein coupling) and indirectly (mediated through PKC control of the signaling system). In addition, IP_3R function is affected by ethanol, by mechanisms that remain to be further characterized. Adaptation to chronic ethanol treatment, on the other hand, appears to involve primarily the IP_3R activation characteristics. Our current evidence supports the concept that the Ca^{2+} modulation of IP_3R function is a potential site affected by the adaptive changes induced by chronic ethanol treatment. The mechanism by which IP_3R function is controlled by Ca^{2+}, both positively and negatively, has not been fully characterized (see Joseph 1997). Different IP_3R isoforms probably vary in the mechanism by which Ca^{2+} interacts with the protein. However, recent evidence (Patel et al. 1998; Levitan 1999) suggests that tightly bound calmodulin may be a necessary component of normal function of type I IP_3R (short form), which is the predominant isoform present in liver. Our studies did not detect differences in the IP_3R isoform pattern in hepatocytes from control and ethanol-fed rats. Hence, it will be of interest to investigate if chronic ethanol treatment affects the Ca^{2+}-calmodulin binding characteristics to type I IP_3R in hepatocytes.

A possible implication of the conclusion that the adaptive compensation to chronic ethanol treatment occurs primarily at the level of the IP_3R is an apparent imbalance in signaling responses to PLC-coupled hormones. Under normal conditions, the activation of PIP_2 hydrolysis by PLC results in two signaling branches, one mediated by DAG targeting the PKC family, the other mediated by IP_3 aimed at generating a Ca^{2+} wave. The selective enhancement of the Ca^{2+} signaling

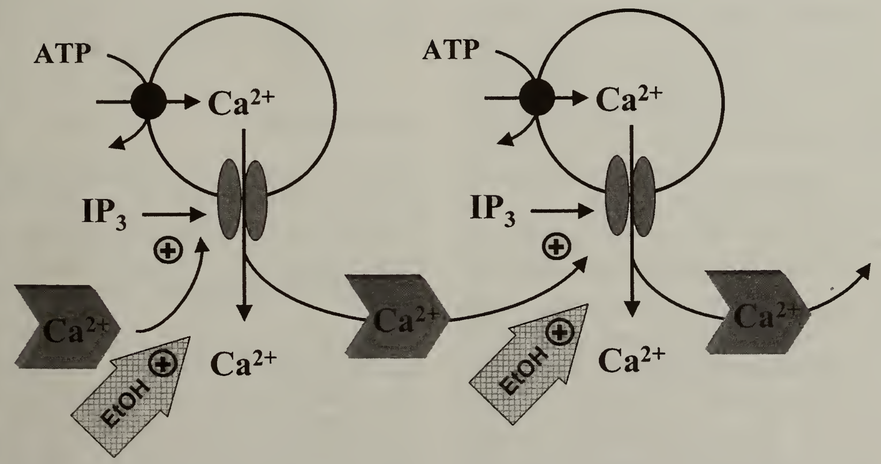

Figure 6. Inositol 1,4,5-trisphosphate (IP_3) receptor modulation by Ca^{2+} as the suggested site of enhancement of IP_3-induced Ca^{2+} release by chronic ethanol (EtOH) treatment. ATP = adenosine triphosphate.

branch at a downstream level mediated by the IP_3R suggests that these cells may have an excessive Ca^{2+} signaling response relative to signals that emanate from PKC activation. Whether this affects the functional responses of the cell in which Ca^{2+} or PKC plays a role remains to be thoroughly investigated. However, it is interesting to note that bile secretion in the liver is characteristically controlled by both Ca^{2+} and PKC-related signals. By contrast, Ca^{2+} regulation of mitochondrial energy metabolism is thought to rely predominantly on changes in $[Ca^{2+}]_i$ (Robb-Gaspers et al. 1998). In fact, it is conceivable that one of the adaptive pressures on the cell is the defective energy metabolism, due to mitochondrial dysfunction in the liver cells from ethanol-fed animals.

It is important to point out that the mechanisms we have explored in this brief overview reflect only part of the possible complexity of ethanol-dependent disturbances of Ca^{2+} related signaling in the liver. We emphasized primarily our studies of the G protein–coupled signaling pathways, but the actions of ethanol with hepatocyte Ca^{2+} signaling pathways are undoubtedly more complex. Additional sites of action relate to the mechanisms by which ethanol interacts with other signaling pathways—for example, tyrosine kinase signaling systems, cyclic adenosine monophosphate (cAMP) signaling systems, other protein kinases, and phosphatases—which have a secondary impact on Ca^{2+} fluxes and thereby affect Ca^{2+} homeostasis. In the intact tissue, there are additional mechanisms of action mediated by cell-cell communication mechanisms that are potential targets of ethanol, for example, signal transfer through gap junctions (Robb-Gaspers and Thomas 1995), or more indirectly, through cytokine release processes that involve Ca^{2+} signals in nonparenchymal cells of the liver and that may affect hormonal response patterns in the parenchymal cells (Goto et al. 1993; Patel et al. 1999*b*). So far, our understanding of these processes is quite limited and the tools to study signaling processes involved in cell-cell communication in the intact tissue are only starting to be developed. In future research on ethanol effects on cell signaling it may be possible to identify the relevant mechanisms at the tissue level.

ACKNOWLEDGMENTS

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Chapter 2

G Protein Mediation of Ethanol Effects on Calcium Channels

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and John Roche, Ph.D.

KEY WORDS: G-protein coupled receptors; ethanol; calcium channel; voltage gated channel; AOD (alcohol or other drug) sensitivity; AODE (effects of AOD use, abuse, and dependence); biological inhibition; cell growth and differentiation; biological activation; laboratory study

Much evidence supports the concept that many of ethanol's actions within the nervous system result from perturbation of the functioning of voltage- and ligand-gated ion channels. Many of these channels are modulated by G proteins and/or exert their effects via the action of G proteins. In this chapter we first present evidence that a component of ethanol-induced inhibition of voltage-gated calcium channels in pheochromocytoma (PC12) cells is mediated by a class of G protein and that the presence of this component is dependent on the differentiation state of the cell. Next, we present data showing how the subunit composition of the voltage-gated calcium channel influences its modulation by G proteins. Thus, alterations in calcium channel subunit composition could conceivably play a role in shifts in ethanol sensitivity of the channel after differentiation and, perhaps, after chronic exposure to the drug. We further discuss the mechanisms that underlie the influence of subunit composition on G protein modulation of the channel, suggesting that the affinity of the channel for the G protein βγ subunit is altered by the presence or absence of the calcium channel β subunit.

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INHIBITION OF L-TYPE CALCIUM CHANNELS BY ETHANOL IN UNDIFFERENTIATED PC12 CELLS

Using whole-cell patch clamp recordings, we established that L-type calcium channels represent the predominant species of voltage-gated calcium channel in undifferentiated PC12 cells and that the ethanol inhibition of calcium current was predominantly by action on this channel type. In these experiments, we used voltage protocols that are diagnostic for various classes of voltage-gated calcium channel. In addition, selective pharmacological blockers were used.

The reduction of current carried through Ca^{2+} channels in the presence of ethanol was independent of the charge carrier; currents carried by Ca^{2+}, Ba^{2+} or Na^{+} were all inhibited to the same extent by 25 mM ethanol. This result suggests that ethanol-induced inhibition of currents does not involve a change in driving force due to an increase in intracellular Ca^{2+} levels. A rise in intracellular Ca^{2+} from internal stores would both decrease the driving force and enhance channel inactivation, which would reduce the currents. Both of these explanations are unlikely because ethyleneglycoltetraacetic acid (EGTA) was present in the patch pipette solution, effectively buffering intracellular Ca^{2+} to ~10 nM. Also, if the effects were due to a change in driving force because of a rise in intracellular Ca^{2+}, then Na^{+} and Ba^{2+} current would be less affected by ethanol than Ca^{2+} currents. Ethanol-induced inhibition of currents carried by any of the three ions does not appear to affect the current-voltage relationship for the channels. The equivalency of ethanol's reduction of current carried by the three ions makes it unlikely that the high-affinity binding sites that constitute the selectivity filter for Ca^{2+} are a site of action for ethanol.

EFFECT OF NERVE GROWTH FACTOR–INDUCED DIFFERENTIATION ON THE ACUTE INHIBITION OF L-TYPE CURRENTS BY ETHANOL

The effects of acute exposure to ethanol on high-threshold, voltage-activated calcium (Ca^{2+}) channels in undifferentiated PC12 cells and in PC12 cells treated with nerve growth factor (NGF) were compared using the nystatin perforated-patch voltage clamp technique. Ethanol inhibition of Ca^{2+} channel currents was examined by exposing single cells to 10, 25, or 50 mM ethanol for 5 minutes. Typically, peak current amplitude evoked from a holding potential (V_h) of –40 mV in an undifferentiated cell was decreased ~40 percent, whereas the amplitude of current at its peak in a NGF-treated cell was decreased by only ~20 percent, by 25 mM ethanol. In both cases, inhibition was reversible but usually required several minutes to return to at least 80 percent of control values. Representative calcium current traces from undifferentiated and NGF-treated cells treated with ethanol can be seen in figure 1.

TIME COURSE OF INHIBITION BY ACUTE EXPOSURE TO ETHANOL

Experiments were performed to determine the time course of ethanol (25 mM) inhibition of current in both cell types. The reduction of peak current amplitude in an undifferentiated cell developed slowly, reaching 16.5 ± 4.4 percent (*n* = 6) by 1–2 minutes and reaching a maximum of 46.3 ± 5.3 percent (*n* = 6) by 4–5 minutes. Inhibition of peak current amplitude by 25 mM ethanol showed only a slight further increase (average 6.9 percent) after an additional 5 minutes (total time exposed to ethanol = 10 minutes). The reduction of peak current amplitude in NGF-treated cells developed even more slowly, reaching 12.2 ± 2.1 percent (*n* = 3) by 4 minutes and reaching a maximum of 21.3 ± 4.1 percent (*n* = 3) by 7 minutes. Inhibition by 25 mM ethanol did not increase appreciably

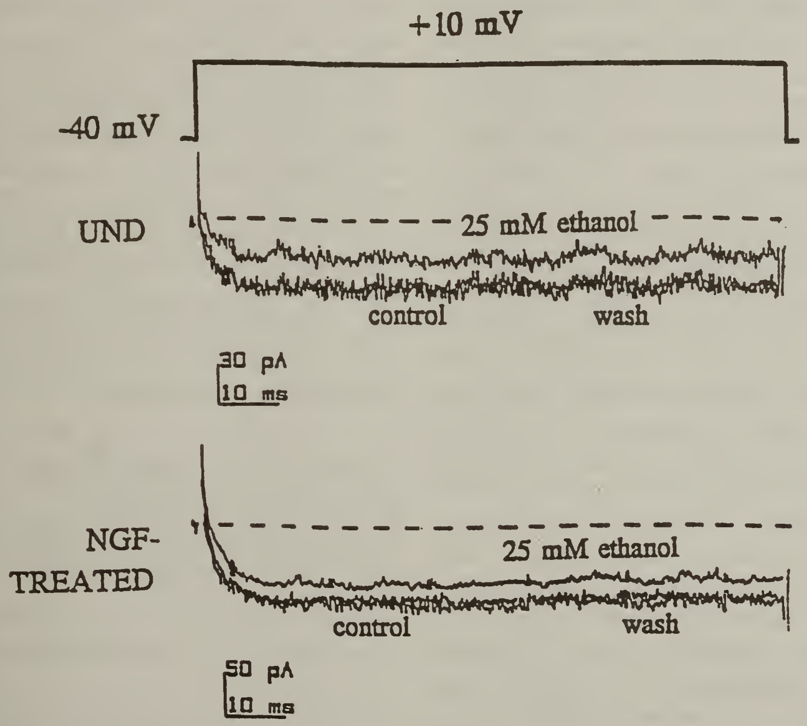

Figure 1. Reversible inhibition of Ca^{2+} currents by acute exposure to ethanol. Representative voltage clamp currents evoked in an undifferentiated (UND) cell (top) and in a nerve growth factor (NGF)–treated cell (bottom) before (control), during, and after (wash) a 5-minute superfusion with ethanol. pA = picoamperes. Reprinted with permission from Mullikin-Kilpatrick, D.; Mehta, N.D.; Hildebrandt, J.D.; and Treistman, S.N. G_i is involved in ethanol inhibition of L-type calcium channels in undifferentiated but not differentiated PC-12 cells. *Molecular Pharmacology* 47:997–1005, May 1995.

(average 3.0 percent) after an additional 3 minutes (total time exposed to ethanol = 10 minutes).

NGF-induced differentiation of PC12 cells is not a simple, immediate process and, over time, results in many morphological as well as intracellular changes. Accordingly, we examined the relationship between the duration of NGF treatment and the reduction in ethanol inhibition. We found that the NGF-related decrease in ethanol sensitivity of the currents occurred in two phases. After a 15-minute exposure to NGF, the reduction by 25 mM ethanol of current amplitude was not different from the reduction of current seen in undifferentiated cells (37.6 ± 7.9 percent, *n* = 5; vs. 45.0 ± 8.8 percent, *n* = 24). However, after 2 hours of exposure to NGF, the reduction of current amplitude in the presence of ethanol fell from 37.6 ± 7.9 percent inhibition to 12.6 ± 4.0 percent inhibition (*n* = 9). Following this first phase of decreased ethanol sensitivity, the reduction of current amplitude returned to the level seen in undifferentiated cells after 5 hours of NGF exposure. Continued exposure to NGF revealed a second phase, which was characterized by a steady decline in the reduction by ethanol of peak current amplitude from 34.7 ± 2.8 percent inhibition (*n* = 3) at 10 hours to 14.5 ± 2.3 percent inhibition (*n* = 6) at 96 hours. This level of inhibition remained constant for up to 240 hours of NGF exposure and was reversible after removal of NGF (see the next paragraph). These results suggest that the effects of NGF on ethanol inhibition of Ca^{2+} channel currents probably involve multiple processes. We found that as early as 15 minutes after NGF exposure, peak current amplitudes were increased about twofold compared with currents in undifferentiated cells and remained at this level throughout the treatment period. The size of the current did not influence the degree of inhibition of current amplitude by ethanol.

We examined whether the NGF-induced decrease in ethanol inhibition of Ca^{2+} channels was reversible. Peak current amplitude evoked in cells treated with NGF for 7–10 days was decreased 16.2 ± 4.8 percent (*n* = 5) by 25 mM ethanol. Currents from cells treated with NGF for 7–10 days and then without NGF for the next 2–5 days were inhibited 41.1 ± 7.8 percent (*n* = 5) by 25 mM ethanol. This inhibition was not significantly different from that observed in undifferentiated cells, indicating that the effects of NGF observed during the second phase were reversible.

EFFECTS OF DIBUTYRYL CYCLIC ADENOSINE MONOPHOSPHATE ON ETHANOL INHIBITION

Dibutyryl cyclic adenosine monophosphate (dbcAMP), a membrane-permeable analog of cyclic adenosine monophosphate (cAMP), causes PC12 cells to extend short cytoplasmic processes and induces many of the same early events as does NGF exposure but does not result in morphological differentiation. To determine if the decrease in the reduction by ethanol of current amplitude observed with NGF treatment was due to cAMP or a cAMP-dependent process, we compared the ability of dbcAMP with that of NGF to affect ethanol inhibition of current.

The first phase of reduced ethanol sensitivity was evident after dbcAMP treatment. After a 2-hour exposure, peak current amplitude in the presence of 25 mM ethanol was reduced by 22.1 ± 3.8 percent ($n = 5$), similar to that seen after a 2-hour exposure to NGF. In contrast with the reduced ethanol inhibition observed after 4 days of NGF treatment, there was no second phase of reduced ethanol sensitivity with dbcAMP treatment. Following a 22-hour exposure to dbcAMP, currents were reduced by 25.3 ± 9.9 percent ($n = 4$) in the presence of ethanol; the reduction by ethanol of current amplitude after a 96-hour exposure to dbcAMP was 55.2 ± 6.2 percent ($n = 5$) and after a 168-hour exposure was 67 ± 3.5 percent ($n = 6$). None of these values was different from the level of inhibition seen in undifferentiated cells. Thus, increased levels of cAMP do not appear to be involved in the longer term effects of NGF on the decrease in ethanol inhibition of Ca^{2+} channels, whereas the effects observed at 2 hours may be influenced by the levels of cAMP.

EFFECTS OF ETHANOL ON THE VOLTAGE-DEPENDENCE OF STEADY-STATE INACTIVATION OF Ca^{2+} CURRENTS

We believe that ethanol inhibition of L-type calcium currents occurs via two mechanisms. First, there is a shift in the steady-state inactivation properties of the channel such that, at a given potential, fewer channels are available for activation in the presence of ethanol. This process appears to be common to both undifferentiated and differentiated PC12 cells. Second, there is a component of inhibition mediated by G_i, which is lost after differentiation. We will first present the evidence for the steady-state shift in inactivation and then present the data exploring the role of G proteins in inhibition in the undifferentiated cells.

The voltage-dependence of Ca^{2+} channel inactivation was examined by varying the V_h and applying the same test pulse in the absence and presence of ethanol in both cell types. Current amplitudes obtained at each V_h were normalized to those obtained at a V_h of −90 mV and were plotted against the V_h (figure 2). Ethanol (25 mM) altered the steady-state inactivation properties of the channels by significantly ($p < 0.05$) shifting the half-maximal inactivation potential ($V_{1/2}$) from −33.2 ± 1.3 to −43.9 ± 2.7 mV in undifferentiated cells ($n = 18$) and from −30.2 ± 1.4 mV to −43.0 ± 1.6 mV in NGF-treated cells ($n = 14$). The $V_{1/2}$ values in the absence of ethanol were not different between cell types, and the slope values (k) in the absence or presence of ethanol were also not different. The shift in $V_{1/2}$ in the presence of ethanol was concentration dependent. In undifferentiated cells ($n = 12$), 50 mM ethanol caused a shift in $V_{1/2}$ that was 2.5-fold greater compared with the shift caused by 25 mM ethanol. Comparable experiments in NGF-treated cells (n = 8) revealed a 1.7-fold increase in the shift in $V_{1/2}$ compared with the shift caused by 25 mM ethanol.

The data described so far represent three major findings: (1) dihydropyridine (DHP)-sensitive channels (i.e., L-type) in NGF-treated cells are less sensitive to the effects of acute ethanol than DHP-sensitive channels in undifferentiated cells, (2) the NGF-induced reduction in ethanol sensitivity of Ca^{2+} channels occurred in two phases, and (3) ethanol produces a hyperpolarizing shift in steady-state inactivation of Ca^{2+} channels, which accounts for a significant portion of the inhibition in both cell types.

INVOLVEMENT OF G PROTEINS IN ETHANOL INHIBITION OF CURRENT

To determine whether calcium currents in our PC12 cells were affected by G proteins, we used the nonhydrolyzable guanosine triphosphate (GTP) analog guanosine-5′-O-(3-thiotriphosphate) (GTP-γ-S). This compound irreversibly activates G proteins by allowing dissociation of the three subunits and preventing their reassociation. When compared with currents in cells dialyzed in the absence of GTP-γ-S, a 10-minute perfusion with 200 µM GTP-γ-S reduced the peak current amplitude in both undifferentiated and NGF-treated

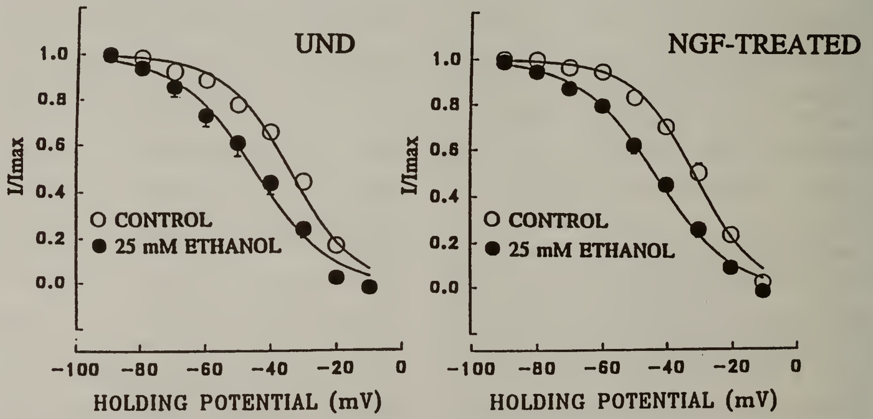

Figure 2. Effects of ethanol on steady-state inactivation of Ca^{2+} channel current. Currents were evoked in the absence and presence of 25 mM ethanol from various holding potentials maintained for 30 seconds, before stepping to either 0 or +10 mV. Ca^{2+} is the charge carrier. Data are normalized to the current obtained at a V_h of -90 mV (mean ± SEM) in undifferentiated (UND) (n = 18) and nerve growth factor (NGF)–treated (n = 14) cells. Error bars are absent when the error is smaller than the symbol. The lines were fitted using a least-squares curve fit according to the Boltzmann equation: $I/I_{max} = 1/(1+\exp((V-V_{1/2})/k))$. Reprinted with permission from Mullikin-Kilpatrick, D., and Treistman, S.N. Inhibition of dihydropyridine sensitive Ca^{++} channels by ethanol in undifferentiated and nerve growth factor-treated PC12 cells: Interaction with the inactivated state. *Journal of Pharmacology and Experimental Therapeutics* 272:489–497, February 1995.

cells by 40 to 60 percent. These results indicate that the activation of a G protein (or more than one) can modulate the calcium currents in both cell types.

Because the inhibition of currents by intracellular infusion of GTP-γ-S did not reach a steady state and continued to increase during the course of an experiment, we could not use this GTP analog to test for G protein involvement in ethanol inhibition. Another compound that can be used to test for G protein involvement is the nonhydrolyzable guanosine diphosphate (GDP) analog guanosine-5′-O-(2-thiodiphosphate) (GDP-β-S). This compound blocks activation of G proteins by competing for the GTP binding site and preventing subunit dissociation. By stabilizing the G proteins in their inactive form, GDP-β-S has been shown to reduce the inhibitory effect of many neurotransmitters on calcium channels.

Ethanol inhibited currents in control undifferentiated cells by 41.6 ± 6.6 percent ($n = 5$), but in cells perfused with 1 mM GDP-β-S, ethanol inhibition was significantly reduced ($p < 0.002$) to 10.7 ± 4.2 percent ($n = 6$). GDP-β-S had no effect on currents in the absence of ethanol. In contrast to the results in undifferentiated cells, GDP-β-S did not produce a decrease in ethanol inhibition in NGF-treated cells. After NGF treatment, ethanol inhibited currents in control cells by 27.7 ± 5.1 percent ($n = 7$), while currents in cells perfused with 1 mM GDP-β-S were inhibited 31.6 ± 7.8 percent ($n = 8$). Higher concentrations of GDP-β-S also had no effect on ethanol inhibition of current in these cells. Collectively, these results indicate that a G protein is involved in ethanol inhibition of calcium currents in undifferentiated cells, but does not appear to be involved in the inhibition in NGF-treated cells.

Other compounds that can be used to verify if a G protein is involved in mediating a drug's effect(s) are fluoride complexes of aluminum. These compounds activate G proteins directly by mimicking the action of the γ PO_4 (gamma phosphate) of GTP, which promotes dissociation of G protein subunits and results in activation of the G protein. These compounds differ from GTP-γ-S because they do not inhibit the reassociation of the G protein subunits. Addition of 5 mM sodium fluoride to the patch electrode caused a reduction in ethanol inhibition from ~40 percent to 13.9 ± 5.6 percent ($n = 4$) in undifferentiated cells. These results support the view that a G protein may act at some point in the activation pathway and is likely to be involved in ethanol inhibition of calcium currents in undifferentiated cells.

IDENTIFICATION OF THE G PROTEIN(S) MODULATING ETHANOL INHIBITION

Many G proteins are present in PC12 cells. We used pertussis toxin (PTX) to help identify the family of G proteins involved in mediating ethanol inhibition. PTX is a bacterial endotoxin that prevents receptor interaction with both the G_i and G_o families of G proteins. PTX catalyzes the transfer of adenosine diphosphate (ADP)-ribose from intracellular NAD^+ to the α subunits of the heterotrimeric complexes of G_i and G_o, thus altering the α subunits, leaving them permanently activated. In undifferentiated cells treated with heat-inactivated PTX, ethanol inhibited currents by 34.3 ± 5.3 percent ($n = 6$), whereas currents in cells treated with active PTX overnight were reduced by only 11.0 ± 6.1 percent ($n = 7$). PTX pretreatment did not reduce ethanol inhibition of currents in NGF-treated cells. These results demonstrate that the G protein(s) involved in ethanol inhibition of calcium current in undifferentiated cells are PTX sensitive.

Various affinity-purified antibodies were used in conjunction with immunoblots to identify the α subunits of the PTX-sensitive G proteins present in plasma membrane preparations from undifferentiated and NGF-treated cells. The $G_i\alpha1\&2$-peptide antibody, which recognizes the α subunit of both G_i1 and G_i2, recognized a 40-kd and a 41-kd protein, respectively, in membranes from both undifferentiated and NGF-treated cells. Qualitatively, there was no difference between the two cell types with respect to the α subunit of either the G_i1 or G_i2 protein. The $G_o\alpha A$-peptide antibody recognized a protein in the 40-kd region, while the $G_o\alpha B$-peptide antibody identified a 39-kd protein in both sets of membranes. When compared with undifferentiated cells, NGF treatment caused an increase in the $G_o\alpha A$ protein and a decrease in the $G_o\alpha B$ protein. Using an antibody directed toward the carboxyl terminus of the β subunit, we found that NGF treatment also produced an increase in this protein when compared with levels found in undifferentiated cells. These results demonstrate that the G proteins present in the two cell types are indistinguishable, but that the levels of some of the G protein subunits change after NGF treatment.

To confirm that the $G_i\alpha1\&2$ antibody was functional, we monitored the effect of the antibody on the inhibition of currents produced by application of 10 μM clonidine. This agonist is selective for the α_2 adrenergic receptor, which can couple to G_i or G_o, and has been shown to inhibit calcium currents in undifferentiated PC12 cells. Internal perfusion of undifferentiated cells with the $G_i\alpha1\&2$ antibody reduced clonidine inhibition from 33.6 ± 10.9 percent ($n = 3$) to 10.7 ± 2.9 percent ($n = 3$). In the absence of clonidine, the $G_i\alpha1\&2$ antibody had no effect on currents.

Similarly, we determined that the $G_o\alpha A$ or B antibodies were functional by examining the inhibition of currents by D-ala-D-leu enkephalinamide (DADLE). This peptide is a selective agonist for the delta opiate receptor, which can also couple to G_o or G_i, and has also been shown to reduce Ca^{2+} currents in undifferentiated PC12 cells. Inhibition of currents by 1 μM DADLE was reduced from 28.1 ± 7.1 percent ($n = 3$) to 2.5 percent (0 and 5 percent, $n = 2$) by the $G_o\alpha B$ antibody. In the presence of the $G_o\alpha A$ antibody, inhibition of currents by DADLE was not reduced.

These results indicate that the $G_i\alpha1\&2$ and $G_o\alpha B$ antibodies can attenuate receptor-mediated inhibition of calcium currents in undifferentiated PC12 cells and can be used to determine which G protein is involved in mediating ethanol inhibition of calcium currents. It is not clear whether the $G_o\alpha A$ antibody is functional in the intact cell. Our finding that the antibody recognized the $G_o\alpha A$ protein by Western analysis suggests that the antibody is functional in vitro.

Using these same antibodies, we identified which PTX-sensitive G protein modulated ethanol inhibition of current. The same dilutions of antibodies used for immunoblots and to study receptor-mediated inhibition of calcium currents were used in these experiments. In an undifferentiated cell perfused with heat-inactivated $G_i\alpha1\&2$ antibody, ethanol inhibited the current by 42.2 ± 2.5 percent (n = 11), which was not significantly different from control values. In contrast, ethanol inhibition was significantly reduced to 16.4 ± 4.6 percent (n = 12) in undifferentiated cells perfused with active $G_i\alpha1\&2$ antibody. In undifferentiated cells, neither heat-inactivated $G_o\alpha A$ or $G_o\alpha B$ nor active $G_o\alpha A$ antibodies had an effect on the currents or the response to ethanol when compared with control cells. The lack of an effect on ethanol inhibition by the active $G_o\alpha A$ antibody may be because the antibody is not functional in the intact cell. A more likely explanation is that the $G_o\alpha A$ protein is not involved in DADLE-mediated inhibition or ethanol inhibition of Ca^{2+} currents. In four out of six cells, the $G_o\alpha B$ antibody had no effect on ethanol inhibition, whereas in two cells, the antibody produced a modest reduction in ethanol inhibition. It is possible that the $G_o\alpha B$ subunit can substitute for $G_i\alpha$ and thus mediate ethanol inhibition of calcium currents, under certain conditions. In NGF-treated cells, neither the $G_i\alpha1\&2$ antibody nor the $G_o\alpha A$ or B antibodies caused any decrease in ethanol inhibition.

As described earlier in this chapter, acute exposure to ethanol alters the voltage-dependence of Ca^{2+} channel inactivation by causing a significant hyperpolarizing shift in the $V_{1/2}$ in both undifferentiated and NGF-treated PC12 cells. To determine whether the $G_i\alpha1$ or 2 protein affected the steady-state inactivation properties of the channels, we internally perfused undifferentiated cells with the $G_i\alpha1\&2$ antibody and measured steady-state inactivation of the channels. Using 10 mM Ca^{2+} as the charge carrier, currents were evoked from various holding potentials (–80, –70, –60, –50, –40, –30, –20 mV), which were maintained for 30 seconds before stepping to +10 mV, in the absence and presence of 25 mM ethanol. Data were normalized to the current obtained at a V_h of –80 mV and the results were fitted using a curve fit routine according to the Boltzmann equation. In the absence of ethanol, the $V_{1/2}$ was unaltered by internal perfusion with either heat-inactivated or active antibody. Exposure to 25 mM ethanol caused an approximate 10-mV hyperpolarizing shift in the $V_{1/2}$ in the presence of either heat-inactivated $G_i\alpha 1\&2$ antibody or active antibody. In addition, the slope values (*k*) were not different between control and ethanol or heat-inactivated antibody and active antibody. These results indicate that the ethanol-induced shift in steady-state inactivation of Ca^{2+} channels is not mediated by the $G_i\alpha 1$ or 2 protein.

Collectively, our results indicate that the difference in ethanol sensitivity of L-type calcium channels in undifferentiated and NGF-treated cells is due to the PTX-sensitive G protein, $G_i\alpha 1$ and/or 2. This protein is involved in ethanol inhibition in undifferentiated cells but not in NGF-treated cells.

A number of factors could be responsible for the loss of the G protein component of channel inhibition after differentiation by NGF. One of the more obvious is a shift in the G protein composition of the cell. Another possibility is that the calcium channel changes in a manner that makes it less receptive to this type of modulation. To test whether this might be a viable mechanism, in principle, we examined the influence of subunit composition on G protein modulation of functional channels formed from three classes of calcium channel α subunit, in either the presence or the absence of the calcium channel β_3 subunit. These studies were performed on calcium channels heterologously formed from messenger ribonucleic acid (mRNA) injected into *Xenopus* oocytes. It should be emphasized that there is currently no evidence that the mechanisms for modulating G protein interactions described in the oocyte studies explain the previously described findings in PC12 cells. However, we feel that juxtaposing these studies is appropriate and provides a testable hypothesis for the manner in which treatments such as differentiation or chronic drug exposure might alter target channels, altering G protein–mediated drug effects.

RESULTS OF OOCYTE STUDIES ON THE INFLUENCE OF CALCIUM CHANNEL SUBUNIT COMPOSITION ON MODULATION BY G PROTEINS

Within the past few years, a variety of Ca^{2+} channel α_1 subunits, as well as several auxiliary subunits, have been cloned. Expression of the α_1 subunit is sufficient for the formation of functional Ca^{2+} channels. Current through α_1 channels can be modulated by co-expression of the auxiliary subunits. The α_2 subunit is thought to reside mostly extracellularly, and has been shown to cause a modest increase in current amplitude when compared with current through α_1 alone. Co-expression of the β subunit with a variety of α_1 clones results in currents with drastically altered characteristics, including a shift in activation of current and peak current amplitudes to more hyperpolarized levels, an increase in the rate of current activation/inactivation, and an increase in current amplitude.

Oocytes were injected with mRNA encoding α_{1A}, α_{1B}, or α_{1C} alone, or in combination with α_{2B} (1:1), or in combination with both α_{2B} and β_3 (1:1:1), and two-electrode voltage clamp currents were recorded. To determine if the Ca^{2+} channel clones used in our experiments were inhibited by G proteins under basal conditions, we injected GDP-β-S into oocytes expressing individually the α_{1A}, α_{1B}, or α_{1C} calcium channels. GDP-β-S binds G proteins and blocks the exchange of GDP for GTP, preventing activation of the G protein, since association with GTP is necessary for the active form of the protein.

Injection of GDP-β-S into oocytes expressing the α_{1A} Ca^{2+} channel subunit without auxiliary subunits caused an approximate twofold increase in current amplitude and an increase in the rate of current inactivation. The α_{1B} channel also demonstrated an approximate twofold increase in current amplitude after GDP-β-S treatment and a similar increase in the rate of current inactivation. In contrast to the results obtained with α_{1A} and α_{1B}, no increase in current amplitude or change in current kinetics was seen after treatment with GDP-β-S in the α_{1C} calcium channel. Representative traces can be seen in figure 3.

To confirm whether the effect of GDP-β-S was due to relief of G protein–mediated inhibition, we used the sulfhydryl-alkylating agent *N*-ethylmaleimide (NEM). NEM at low doses (0.1–0.2 mM) has been shown to uncouple certain classes of G proteins from their receptors. A 2-minute application of NEM also caused a twofold increase in current amplitude in both α_{1A} and α_{1B} currents. We conclude from these data that there is a tonically active G protein population in *Xenopus* oocytes that inhibits the α_{1A} and α_{1B} but not the α_{1C} calcium channels. The inhibition is mediated by an NEM-sensitive G protein population and affects current kinetics as well as current amplitude.

Recently, the α_{1B} calcium channel was found to co-purify with the α_{2B} and β_3 subunits. Using this subset of Ca^{2+} channel subunits to test for tonic G protein inhibition, we co-expressed either α_{1A} or α_{1B} with either α_2 or $\alpha_2\beta_3$. Co-expression of α_{2B} with α_{1A} or α_{1B} had no discernible effect on current

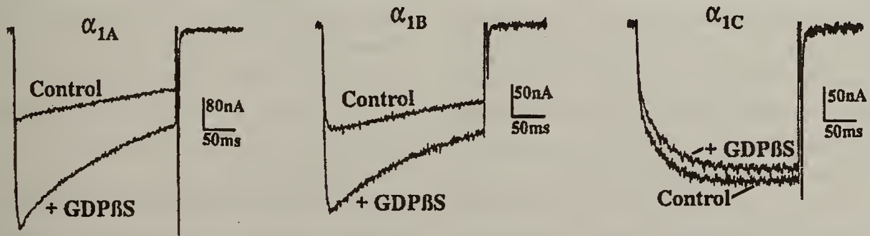

Figure 3. Effect of G protein inactivation on three different classes of α_1 subunit expressed individually in *Xenopus* oocytes. Ba^{2+} currents recorded before and after injection of guanosine-5′-O-(2-thiodiphosphate) (GDP-β-S) in oocytes expressing either α_{1A}, α_{1B}, or α_{1C}. nA = nanoamperes. Reprinted with permission from Roche, J.P.; Anantharam, V.; and Treistman, S.N. Abolition of G protein inhibition in α1A and α1B calcium channels by co-expression of the β3 subunit. *FEBS Letters* 371:43–46, 1995.

amplitude, kinetics, or voltage-dependence of the baseline currents when compared with α_{1A} or α_{1B} alone. Co-expression of the $\alpha_2\beta_3$ combination with α_{1A} caused an approximately fourfold increase in current amplitude, a shift in the voltage-dependence of activation to more hyperpolarized levels, and an apparent increase in the rate of current inactivation when compared with currents through α_{1A} expressed alone. Co-expression of $\alpha_2\beta_3$ with α_{1B} caused effects nearly identical to co-expression with $\alpha_{1A.}$

The $\alpha_{1A}\alpha_{2B}$ combination showed a significant increase in current amplitude and in decay rate of the inactivating current after both GDP-β-S and NEM treatments, similar to the effects of GDP-β-S treatment on α_{1A} alone. However, when the β_3 subunit was added to this combination, the effects of GDP-β-S or NEM on both current amplitude and kinetics were abolished. The results were similar when the α_2 and $\alpha_2\beta_3$ combinations were expressed with α_{1B}. Neither GDP-β-S nor NEM had a significant effect on current kinetics after co-expression of the β_3 subunit. Thus, co-expression of the β_3 subunit can block inhibition mediated by the tonically active G proteins on α_{1A} and α_{1B} calcium channels. In addition, it appears that the α_2 subunit has no effect on this G protein–mediated inhibition.

One type of G protein inhibition that is commonly seen in neurons is dependent on the membrane potential of the cell. Strong depolarizations are thought to temporarily uncouple the activated G protein from the Ca^{2+} channel, producing larger currents after depolarizing prepulses, by relieving the inhibitory effect of the G proteins. To test whether the tonic inhibition of α_{1A} and α_{1B} seen in *Xenopus* oocytes displayed similar voltage-dependence, we used a voltage protocol in which two test pulses were separated by a period in which the channels were allowed to recover from inactivation. Shortly before the second test pulse, a strong depolarizing prepulse was given. The currents elicited by the two test pulses were then compared.

Currents through both α_{1A} and α_{1B} channels could be facilitated with a strong depolarizing prepulse using the prepulse voltage protocol. Facilitation of the α_{1A} and α_{1B} currents was abolished after GDP-β-S or NEM treatments. In addition, no current facilitation was observed when the β_3 subunit was co-expressed with the α_{1A} or α_{1B} subunits. We conclude from these data that the inhibition of α_{1A} and α_{1B} currents by the tonically active G protein population is voltage dependent, similar to G protein–mediated inhibition described previously in a variety of neurons. Additionally, lack of facilitation after co-expression of the β_3 subunit is consistent with our conclusion that co-expression of the β_3 subunit abolishes tonic G protein inhibition of these channels.

The voltage-dependence of inhibition that we observed for α_{1A} and α_{1B} currents, coupled with the persistence of G protein inhibition in the presence of the Ca^{2+} chelator, BAPTA (1,2-bis(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid), makes it likely that the G protein inhibition occurring is of the membrane-delimited type. In this model, the G protein is believed to interact directly with the channel. Since the α_1 subunit is sufficient for G protein–mediated inhibition of α_{1A} and α_{1B} currents, the G protein presumably interacts directly with this subunit.

It is not clear whether the G protein–mediated component of ethanol inhibition of calcium channels in the undifferentiated PC12 cells represents tonic inhibition, comparable to that which we demonstrated in the transfected oocyte, although the lack of effect of GDP-β-S suggests otherwise. To better understand the mechanisms underlying G protein inhibition of calcium channels, we co-transfected muscarinic acetylcholine receptors, along with calcium channel subunits, allowing significantly more control over the activation of the G protein population than was possible by manipulation of the tonically active G protein population. We could then determine the modulatory effect of the Ca^{2+} channel β_3 subunit on M_2 muscarinic receptor–activated G protein inhibition, and whether the β_3 subunit modulates the G protein sensitivity of α_{1A} and α_{1B} currents equivalently.

Our results indicate that co-expression of the calcium channel β_3 subunit differentially modulates both the magnitude and voltage-dependence of M_2 receptor–induced G protein inhibition of α_{1A} and α_{1B} Ca^{2+} currents. The magnitude of inhibition, previously equivalent for α_{1A} and α_{1B} Ca^{2+} currents at moderate depolarizations, becomes greater for $\alpha_{1B}\beta_3$ current compared with $\alpha_{1A}\beta_3$ currents. In addition, G protein inhibition of $\alpha_{1A}\beta_3$ channels becomes less voltage dependent, while inhibition of $\alpha_{1B}\beta_3$ channels becomes more voltage dependent than α_{1A} and α_{1B} channels are in the absence of the β subunit.

The Ca^{2+} channel β_3 subunit dramatically decreased the duration of the prepulse necessary for maximal relief of muscarinic inhibition, from ~160 ms to less than 20 ms; a single exponential fit to the data revealed a decrease in the time constant of relief by a voltage step to +100 mV, from 67 ms in the absence of β_3 auxiliary subunit to 6.9 ms after co-expression of the Ca^{2+} channel β_3 subunit. A similar reduction in the duration and voltage necessary to produce relief of inhibition was produced by co-expression of the G protein βγ subunit, strongly suggesting that the relief of muscarinic inhibition was a result of increased voltage-dependent dissociation of the G protein βγ subunit. Figure 4 shows the voltage protocols used in these experiments, as well as the modulation of prepulse inhibition by the calcium channel. We conclude that the Ca^{2+} channel β_3 subunit reduces the magnitude of muscarinic-induced G protein inhibition of α_{1B} Ca^{2+} channels by enhancing the rate of dissociation of the G protein βγ subunit from the Ca^{2+} channel α_{1B} subunit.

In this chapter we have presented data showing that a component of the ethanol inhibition of the L-type voltage-gated calcium channel in the undifferentiated PC12 cell is mediated by G_i, and that this component is lost after NGF-induced differentiation of the cell. We have also shown that the subunit composition of the voltage-gated calcium channel can influence the G protein modulation of the channel, providing a potential mechanism for the NGF-induced switch. Further experimentation is necessary to determine whether this mechanism is, indeed, operational in this particular instance, as well as in other examples of plasticity, such as the development of tolerance of a channel to the action of a drug, after chronic exposure to the drug. For more detailed analysis of the data on which this chapter was based, see Mullikin-Kilpatrick and Treistman 1994*a*, 1994*b*, 1995; Mullikin-Kilpatrick et al. 1995; Roche and Treistman 1998*a*, 1998*b*; Roche et al. 1995.

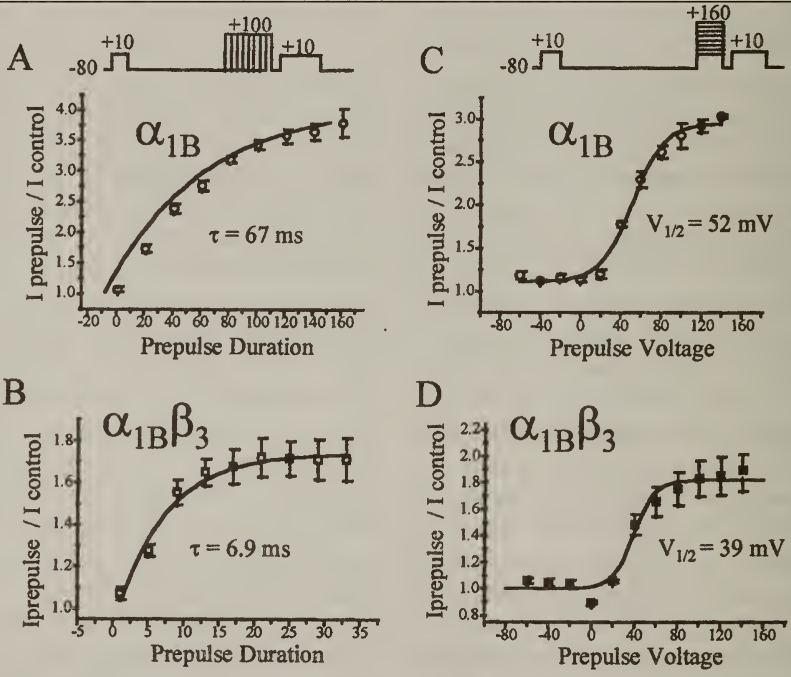

Figure 4. Modulation of time- and voltage-dependency of prepulse facilitation by the Ca^{2+} channel β_3 subunit. (A and B) Facilitation of current amplitude after G protein inhibition induced by application of acetylcholine. The prepulse was +75 mV for varying periods of time, as indicated. The test potential was +10 mV. The data were fit with a single exponential, revealing time constants of 67 ms for the α_{1B} currents and 6.9 ms for the $\alpha_{1B}\beta_3$ currents. (C and D) Facilitation of current amplitude with a 75-ms prepulse of varying voltage, as indicated. The test potential was +10 mV. These data were fit with a Boltzmann curve, revealing $V_{1/2}$ values of 52 mV for the α_{1B} currents, and 39 mV for the $\alpha_{1B}\beta_3$ currents. Reprinted with permission from Roche, J., and Treistman, S.N. Ca^{2+} channel β_3 subunit enhances voltage-dependent relief of G protein inhibition induced by muscarinic receptor activation and $G_{\beta\gamma}$. *Journal of Neuroscience* 18:4883–4890, 1998.

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Chapter 3

Proteasomal Proteolysis and Inositol Trisphosphate Receptors: Targets for Ethanol Action

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KEY WORDS: inositol 1,4,5-trisphosphate; calcium channel; cell signaling; chronic AODE (effects of AOD [alcohol or other drug] use, abuse, and dependence); biological inhibition; immunoblotting; enzymes

SCOPE OF STUDY

It is well established that chronic ethanol consumption impairs protein catabolism in the liver and is associated with an increased accumulation of proteins within the hepatocyte (Lieber 1980; Poso 1987). In the past, it was generally believed that the lysosome played a major role in the proteolysis of most cellular proteins, and therefore the focus of interest in studies of ethanol on protein catabolism lay in characterizing effects of ethanol on lysosomes and lysosomal enzymes. In recent years it has become apparent that the bulk of short-lived and long-lived cellular proteins may be turned over in cells by the ubiquitin-proteasome pathway (Rock et al. 1994; Lee and Goldberg 1998). The effects of ethanol on this proteolytic pathway have not been as well defined.

Our laboratory is interested in studying the structure, function, and regulation of inositol trisphosphate receptors (IP_3Rs). This family of ubiquitous, integral membrane proteins are Ca^{2+} channels located in endoplasmic reticulum (ER) membranes and play a central role in Ca^{2+} signaling occurring in response to agonists that provoke inositol-lipid breakdown in activated cells (reviewed in Joseph 1996; Patel et

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al. 1999). Wojcikiewicz and colleagues have identified several cell types that show an enhanced degradation of this protein in response to chronic stimulation by agonists that generate IP_3 (Wojcikiewicz and Nahorski 1991; Wojcikiewicz et al. 1994; Wojcikiewicz 1995; Wojcikiewicz and Oberdorf 1996). We have extended these studies and shown that degradation of IP_3Rs involves the ubiquitin-proteasome pathway (Bokkala and Joseph 1997). In this chapter, we summarize studies from our laboratory on the effects of chronic ethanol treatment on Ca^{2+} signaling, IP_3R protein levels, and the activity of various proteolytic pathways in WB cells (a rat liver epithelial cell line). We discuss the possibility that a generalized impairment of proteasomal protease activity is induced by chronic ethanol treatment and that the resulting elevation in the levels of important regulatory proteins may play a role in the genesis of alcoholic liver injury.

Ca^{2+} SIGNALING AND CHRONIC ETHANOL TREATMENT

The mechanisms underlying the perturbation of Ca^{2+} signaling in hepatocytes isolated from ethanol-fed animals have been actively investigated by Hoek and colleagues (see chapter 1). In our experimental system, confluent WB cells were exposed to 50–150 mM ethanol for 48 hours. Single-cell imaging studies were carried out on fura-2 loaded WB cells in the absence of extracellular Ca^{2+}. Under these conditions the ethanol-treated WB cells showed an enhanced response to stimulation by angiotensin II (Ang II), vasopressin, or bradykinin (Bokkala et al. 1999). Measurements made in [^3H]inositol-labeled cells suggested that none of these changes could be attributed to enhanced generation of IP_3. Ethanol-treated WB cells also showed an enhanced Ca^{2+} mobilization response to subsaturating doses of IP_3 added directly to permeabilized cells.

These observations indicated that ethanol treatment modified intracellular Ca^{2+} mobilization and prompted additional studies to quantify the levels of immunoreactive IP_3Rs in WB cell lysates. This particular cell type contains the type I and type III isoforms of IP_3Rs, and the levels of both isoforms were found to increase by 60 to 100 percent in response to ethanol treatment. We were unable to detect any effects of ethanol treatment on messenger ribonucleic acid (mRNA) levels for type I IP_3Rs. These results suggest that the inhibitory effects of ethanol may be exerted on the proteolytic pathway(s) involved in IP_3R degradation.

PATHWAYS OF IP_3R PROTEOLYSIS

Both the lysosomal and proteasomal pathways appear to be involved in regulating the turnover of IP_3Rs. In unstimulated cells the half-life of IP_3Rs is estimated to be 8–12 hours (Joseph 1994; Wojcikiewicz et al. 1994). A marked acceleration of this rate occurs in many cell types in response to chronic stimulation with Ca^{2+}-mobilizing hormones (Wojcikiewicz and Nahorski 1991; Wojcikiewicz et al. 1994; Wojcikiewicz 1995; Wojcikiewicz and Oberdorf 1996; Bokkala and Joseph 1997; Sipma et al. 1998). In the case of WB cells, both type I and type III IP_3R isoforms are decreased in response to stimulation by Ang II, but not in response to stimulation by less potent Ca^{2+} mobilizing hormones (e.g., vasopressin). The down-regulation of IP_3Rs occurs over a timeframe of 4–6 hours and is accompanied by an inhibition of IP_3-mediated Ca^{2+}release (Bokkala and Joseph 1997). Treatment of WB cells with NH_4Cl or chloroquine, which are lysosomal protease inhibitors, causes an increase in basal IP_3R levels but does not prevent Ang II–mediated IP_3R down-regulation. In contrast, treatment of WB cells with lactacystin or ALLN (*N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal), which are proteasomal protease inhibitors, does not elevate IP_3R levels but does prevent Ang II–induced IP_3R down-regulation.

These data suggest that the basal turnover of IP_3Rs occurs via a lysosomal pathway, but that an additional pathway of IP_3R degradation can be activated by chronic stimulation with Ca^{2+}-mobilizing hormones (Bokkala and Joseph 1997). The activation of this latter pathway appears to be linked to those hormones that can produce an elevation of IP_3, which is sustained for several hours (Wojcikiewicz et al. 1994; Bokkala and Joseph 1997). It has been proposed that a sustained activation of the IP_3R may induce a conformation of the protein that renders it susceptible to degradation (Wojcikiewicz et al. 1994). Such a conformational change may be associated with exposure of ubiquitination sites. Most proteins are marked for proteasomal hydrolysis by attachment of ubiquitin chains. Using anti-ubiquitin antibodies, we have demonstrated that the IP_3R shows enhanced ubiquitination during Ang II–mediated IP_3R degradation (Bokkala and Joseph 1997). The ubiquitination of IP_3R has now been confirmed in other cell types (Oberdorf et al. 1999; Wojcikiewicz et al. 1999*b*).

EFFECT OF ETHANOL TREATMENT ON LYSOSOMAL PROTEOLYSIS

Our data suggest that the enhancement of agonist-mediated Ca^{2+} signaling observed in chronic ethanol–treated WB cells may be due to enhanced IP_3-mediated Ca^{2+} release resulting from elevated levels of IP_3R protein. The mechanism by which ethanol treatment leads to increased levels of IP_3R protein is unclear. The behavior of WB cells exposed to ethanol is superficially similar to the effects of lysosomal protease inhibitors described above. In particular, neither treatment modifies the Ang II–mediated down-regulation pathway (Bokkala and Joseph 1997; Bokkala et al. 1999). There is substantial evidence in the literature that hepatic lysosomal proteolysis is inhibited by ethanol (Poso and Hirsimaki 1991; Donohue et al. 1994; Kharbanda et al. 1995, 1996, 1997). Lysosomal and endocytic vesicles show an alkalinization of intravesicular pH in response to the acute addition of ethanol. The alkalinization of these compartments is maintained when they are isolated from ethanol-fed animals (Kharbanda et al. 1997). The increase in pH of these compartments may lead directly to an inhibition of protease activity and may also perturb trafficking of key lysosomal enzymes (Kharbanda et al. 1996).

Recently, it has been shown that the acute inhibitory effects of ethanol or acetaldehyde on proteolysis in the perfused rat liver is associated with osmotic swelling caused by activation of the Na-K-2Cl co-transporter (Vom Dahl and Haussinger 1998). The drug bumetanide, which blocks the Na-K-2Cl co-transporter, also blocks the inhibitory effects of ethanol or acetaldehyde on proteolysis. On this basis, it has been suggested that osmotic swelling of hepatocytes promoted by ethanol is an early step in the sequence of events that leads to the alkalinization of lysosomal compartments (Vom Dahl and Haussinger 1998). None of the parameters related to changes in osmotic swelling or intralysosomal pH have been systematically examined in WB cells, and it remains a possibility that inhibition of lysosomal proteolysis may contribute to the mechanism by which ethanol increases the levels of IP_3R protein.

EFFECT OF ETHANOL TREATMENT ON PROTEASOMAL PEPTIDASE ACTIVITY

The proteasome is a large multicatalytic protease complex comprising 7 α and 7 β subunits arranged as four stacked rings in a cylindrical molecule. This functional core is referred to as the “20S complex.” The attachment of an additional set of regulatory proteins to the 20S complex forms the 26S proteasome, which, unlike the 20S complex, requires ubiquitinated substrate and adenosine triphosphate for full activity (reviewed in Coux et al. 1996; Baumeister et al. 1998). The β subunits of the proteasome catalyze three distinct proteolytic activities: chymotrypsin-like, trypsin-like, and peptidylglutamyl peptide hydrolyzing (PGPH) activities. These activities can be measured using specific fluorogenic substrates (Shibatini et al. 1996).

We examined the peptidase activities of the proteasome in membrane and cytosol fractions of WB cells treated with Ang II for 6 hours. Under these conditions we observed an approximately twofold elevation of the chymotrypsin-like and trypsin-like activity in the cytosol and membrane-associated fractions of Ang II–treated cells, with no difference in the PGPH activity (Bokkala and Joseph unpublished observation). Control experiments, in particular the use of the specific inhibitor lactacystin, provided convincing evidence that the chymotrypsin and trypsin-like peptidases being measured in the crude fractions are bona fide activities of the proteasome. These studies suggest that conditions that lead to IP_3R degradation by the proteasomal pathway appear to be associated with a selective activation of proteasomal peptidases. It was therefore of interest to examine if conditions that lead to increased levels of IP_3Rs in WB cells (e.g., chronic ethanol exposure) were associated with an inhibition of proteasomal peptidases. The results of these experiments are shown in figure 1. The chymotrypsin-like peptidase activity of the cytosol and crude membrane fraction of ethanol-treated WB cells was inhibited by 60 percent and 40 percent, respectively (Bokkala et al. 1999). We were unable to detect any difference in the trypsin-like peptidase activity in the cytosol fraction, although a small effect was observed in the membrane fraction. Ethanol treatment had no effect on PGPH activity (Bokkala et al. 1999).

Recently, there have been two reports showing that ethanol feeding in rats inhibits proteasomal peptidase activities. Donohue and colleagues (1998) reported a 35 to 40 percent inhibition of the chymotrypsin- and trypsin-like activities of the proteasome measured in the cytosol and crude homogenates prepared from rats given ethanol by intragastric administration. The PGPH activity was not affected under these conditions. Immunoreactive subunits of the 20S complex were not changed by ethanol treatment. Fataccioli and colleagues (1999) made similar observations but also noted that PGPH activity was inhibited by ethanol. The authors of both studies noted that the ethanol-induced changes in proteasomal peptidase activity were not observed when the livers were isolated from rats fed a Lieber-DeCarli ethanol diet. They attributed the differential effects of ethanol on proteasomal peptidases in the two feeding models to the greater extent of oxidative damage to the liver seen when alcohol is administered by intragastric infusion (Rouach et al. 1997), and they suggested that alcohol-derived acetaldehyde may directly modify the proteasome complex (Donohue et al. 1998) or generate peptide-aldehyde inhibitors (Fataccioli et al. 1999). Although the detailed mechanism remains to be evaluated, the results from the studies on ethanol-fed rats

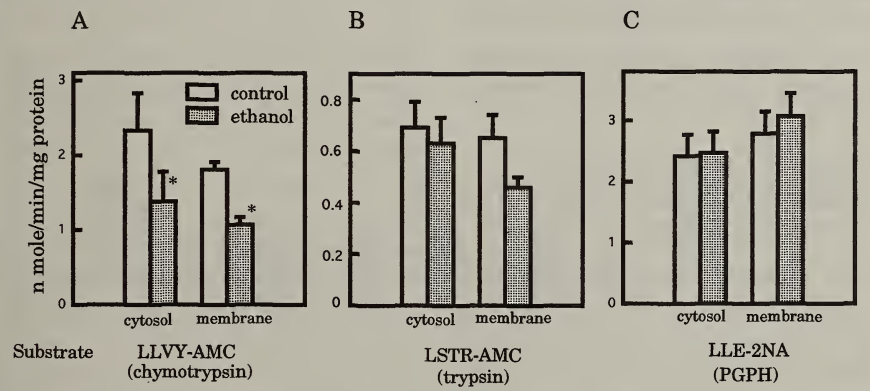

Figure 1. The effect of chronic ethanol treatment on proteasomal peptidase activities in cytosol and membrane fractions from WB cells. WB cells were pretreated with 150 mM ethanol for 24 hours. Control and ethanol-treated cells were fractionated into crude cytosol and membrane fractions and assayed for proteasomal peptidase activity using aminomethyl coumarin (AMC)– or napthylamine (NA)–labeled peptide substrates. The protease activity of the membrane fractions was measured in the presence of 0.05 percent sodium dodecyl sulfate. The activity is expressed as nanomoles of product formed per minute per milligram of protein. The data are the means ± SEM from three independent experiments. *$p < 0.05$. LLVY-AMC = Leu-Leu-Val-Tyr-7-amido-4 methyl coumarin; LSTR-AMC = *N*-t-butoxycarbonyl(Boc)-Leu-Ser-Thr-Arg-7-amido-4-methyl coumarin; LLE-2NA = benzyloxycarbonyl-Leu-Leu-Glu-β-naphthylamide.

are in broad agreement with our findings in WB cells and suggest that chronic ethanol administration may impair the function of the multicatalytic proteasome.

Whether a 40 to 60 percent sustained inhibition of proteasomal peptidase activity is linked to the elevation of IP_3R protein remains to be established. The acute addition of proteasomal inhibitors such as lactacystin or ALLN for 90 minutes does not elevate basal IP_3R levels in WB cells. It is possible that the proteasomal pathway also participates in the basal turnover of IP_3Rs or that ethanol treatment itself may divert IP_3R protein to proteasomal pathways that use a chymotrypsin-like peptidase. The finding that Ang II–mediated down-regulation of IP_3Rs is unaffected by ethanol treatment also suggests that the chymotrypsin-like activity stimulated by Ang II treatment must be different from the chymotrypsin-like activity inhibited by ethanol treatment.

EFFECT OF ETHANOL TREATMENT ON UBIQUITIN CONJUGATION

Even under unstimulated conditions, a small fraction of IP_3Rs in WB cells are ubiquitinated. Maximal ubiquitination of IP_3Rs can be observed when degradation is enhanced by Ang II and when proteasomal activity is blocked (Bokkala and Joseph 1997; Oberdorf et al. 1999). We have not as yet determined if ubiquitinated IP_3Rs remain functional, nor have we assessed the ubiquitination status of IP_3Rs in ethanol-treated WB cells. However, there have been two studies that have examined the effects of chronic ethanol feeding on the status of ubiquitin conjugates in liver.

The first study, by Born and colleagues (1996), used a competitive enzyme-linked immunosorbent assay (ELISA) to show a 40 to 75 percent elevation of ubiquitin levels in lysates prepared from ethanol-fed rats. This difference could not be detected by immunoblotting with anti-ubiquitin antibodies, and there was also no difference in the capacity of lysates to conjugate $[^{125}I]$ubiquitin from control rats or rats fed a Lieber-DeCarli ethanol diet (Born et al. 1996). The effects of ethanol on the levels of ubiquitinated proteins in the gastric infusion model have not been reported. The second study, by Zhang-Gouillon and colleagues (1998), used drug-primed mice to feed ethanol intragastrically and noted the appearance of Mallory bodies. These structures are found in injured hepatocytes and are formed from aggregated and ubiquitinated intermediate filament proteins (Kachi et al. 1993; Zhang-Gouillon et al. 1998). It has been proposed that impaired proteasomal degradation of misfolded cytokeratin may lead to its deposition as ubiquitinated aggregates in Mallory bodies (Zhang-Gouillon et al. 1998). There are several other examples in the literature where oxidative stress has been linked to inhibition of proteasomal activity and the accumulation of ubiquitinated substrates (Conconi et al. 1996; Shang et al. 1997; Figueiredo-Pereira et al. 1998). In particular, a number of neurodegenerative disorders are associated with the accumulation of ubiquitinated protein aggregates (reviewed in Alves-Rodrigues et al. 1998).

A MODEL OF IP_3R DEGRADATION IN THE ENDOPLASMIC RETICULUM

Figure 2 shows a model of IP_3R degradation that incorporates both experimental observations and several speculations that remain to be experimentally tested. The model distinguishes two separate pathways of degradation, which we refer to as "regulated" and "basal" pathways. The regulated degradation pathway is activated in response to chronic stimulation with a Ca^{2+}-mobilizing agonist. In the model, a prolonged elevation of IP_3 leads to a conformational change in the receptor, which results in polyubiquitination of the IP_3R protein. Several studies have shown that IP_3R down-regulation can also be induced by sustained activation of phospholipase Cβ produced by constitutively active mutants of $G_q\alpha$ (Honda et al. 1995; Lobaugh et al. 1996; Quick et al. 1996). In addition, the observation that mutant IP_3Rs defective in ligand binding do not undergo agonist-mediated down-regulation also lends support to the importance of an IP_3-induced conformational change as an initial trigger for degradation (Wojcikiewicz et al. 1999*b*). The actual enzyme(s) involved in ubiquitin attachment to IP_3Rs are not known. A number of candidate gene products involved in the ubiquitination of yeast ER proteins have been identified (Hampton et al. 1996; Biederer et al. 1997;

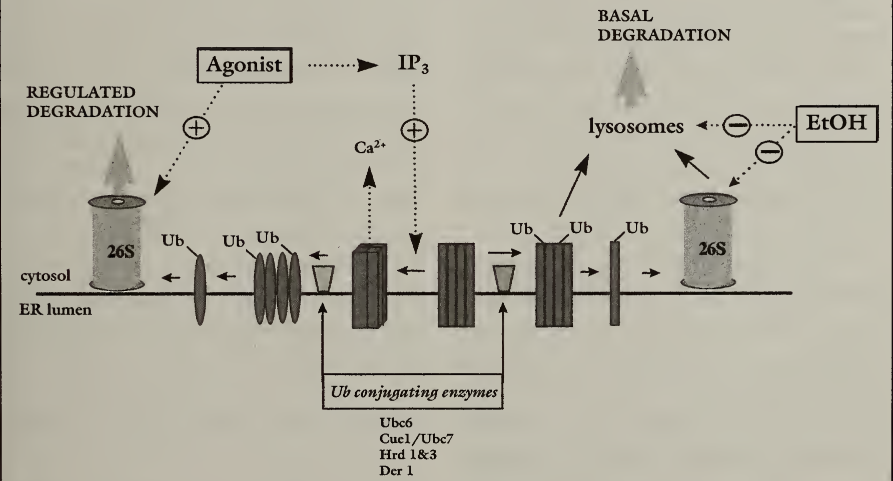

Figure 2. Model of inositol trisphosphate receptor (IP_3R) degradation in the endoplasmic reticulum (ER). The IP_3R is shown as a tetrameric structure, which undergoes a conformational change in response to IP_3 that results in Ca^{2+}mobilization from the lumen of the ER. Prolonged IP_3 elevation caused by agonists may induce a different conformational change that results in ubiquitin conjugation to the protein, disassembly into monomers, and proteasomal proteolysis. Basal degradation may also involve the attachment of ubiquitin to the protein, but at different sites. Both lysosomes and proteasomes are thought to be involved in this pathway, and chronic ethanol (EtOH) treatment is shown as inhibiting both these pathways.

Bordallo et al. 1998). Human and mouse homologues of two of these proteins (UBC6 and UBC7) have also been cloned (Katsanis and Fisher 1998; Tiwari and Wiessman 1998).

In the model, we have focused attention on a pool of proteasomes that are bound to the ER membrane. The association of a small pool of proteasomes with the ER has been demonstrated, and there is some evidence to suggest that their subunit composition and/or regulatory properties may be distinct (Palmer et al. 1996). The model also incorporates our findings that Ca^{2+}-mobilizing agonists can activate proteasomal peptidase activity by an as yet unknown mechanism. The IP_3R is a large tetrameric protein (> 10^6 dalton) and would undoubtedly have to be disassembled, unfolded, and deglycosylated prior to being threaded through the relatively narrow entrance aperture of the proteasome. The details of how this is accomplished and, in particular, how this stably integrated channel protein is removed from the membrane environment remains to be investigated.

Under basal conditions the levels of IP_3 are not elevated, and therefore most of the IP_3Rs would be in an inactive (closed) state. However, low levels of ubiquitinated IP_3R protein are still detected under these basal conditions (Bokkala and Joseph 1997; Oberdorf et al. 1999). It is possible that the attachment of ubiquitin to sites in the IP_3R molecule (distinct from those occupied during regulated degradation) also targets the receptor for turnover under basal conditions. The experimental evidence suggests that this pathway involves lysosomes (Bokkala and Joseph 1997). Other ER resident membrane proteins, such as cytochrome P-450_{2E1} (CYP2EI), are thought to enter lysosomes by the process of macroautophagy (Masaki et al. 1987; Ronis et al. 1991; Cuervo and Dice 1998), and ubiquitinated IP_3Rs may share the same fate. A recent study has shown that a ubiquitin-like molecule is used in a conjugation system to target yeast proteins for autophagy (Mizushima et al. 1998). It is also possible that the concerted action of the proteasome and the lysosome is involved in basal IP_3R degradation. In any case, the machinery involved in facilitating the basal turnover of inactive IP_3Rs is viewed as being different from that involved in down-regulating activated IP_3Rs. The differences may lie in the sites on the IP_3R molecule that are conjugated to ubiquitin, the recognition properties of the ubiquitin conjugating enzymes themselves, or the subunit composition of the pool of proteasomes involved in basal IP_3R turnover. Based on the experimental evidence summarized above, chronic ethanol treatment is shown as inhibiting several steps, including lysosomal proteolysis and selective proteasomal peptidase activities. A third possibility, which remains to be investigated, is that chronic ethanol treatment can inhibit the mechanism targeting the IP_3Rs to the basal degradation pathway.

OTHER SUBSTRATES OF THE UBIQUITIN-PROTEASOME PATHWAY LINKED TO ALCOHOLIC LIVER CELL INJURY

Oxidative stress and the generation of reactive oxygen species (ROS) may exert their effects on the ubiquitin-proteasome pathway in two ways. First, the formation of adducts of ROS with substrate proteins may lead to their unfolding and result in their aberrant ubiquitination and aggregation. Second, oxidative stress may directly suppress the catalytic activity of the proteasome. If the deleterious effects of chronic alcohol consumption involve these mechanisms, we would anticipate that the turnover of a large number of proteins would be affected. In this context, perhaps the most important substrate protein in liver is CYP2E1. It is well established that this enzyme plays a key role in the metabolism of ethanol and the generation of ROS, and as such is very important in the pathogenesis of alcoholic liver injury (Lieber 1997). Like the IP_3R, this protein is also an integral membrane protein of the ER, with the bulk of the molecule exposed to the cytosol. The rapid turnover of CYP2E1 is mediated by the proteasomal pathway, and it has been proposed that substrates of CYP2E1 (e.g., ethanol) may stabilize the protein and thereby inhibit its degradation (Roberts et al. 1995; Roberts 1997; Yang and Cederbaum 1997). Whether CYP2E1 is also ubiquitinated has not been firmly established. There are studies that have shown that the protein can be ubiquitinated (Roberts et al. 1995). However, Roberts (1997) has shown that purified 20S complex can degrade CYP2E1 in microsomal membranes incubated in the presence of $CC1_4$ with no requirement for ubiquitination. In any event, the overall effect of ethanol in stabilizing CYP2E1 and inhibiting proteasomal peptidases leaves the hepatocyte with an impaired ability to remove an enzyme generating oxidative damage in the cell.

Apolipoprotein B-100 (ApoB-100) is an essential component of very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) secreted by the liver. Approximately 20 to 50 percent of newly synthesized ApoB-100 is destroyed by a proteasomal degradation pathway in the ER (Yeung and Chan 1998), and inhibition of this degradation pathway increases the fraction of ApoB-100 secreted from HepG2 cells (Zhou et al. 1998). Hence, an inhibition of proteasomal activity induced by chronic ethanol consumption would be expected to result in an increased hepatic production of lipoproteins (Frohlich 1996).

An important function of proteasomal proteolysis is to generate small antigenic peptides that are displayed on the cell surface by major histocompatibility complex (MHC) class I molecules. Foreign peptides presented in this manner are recognized and destroyed by cytolytic T lymphocytes. The biosynthesis and assembly of the MHCs in the ER itself involves the proteasomal pathway, which is used to remove misassembled or incompletely assembled MHCs (Ploegh 1998). Changes in the activity of selective proteasomal peptidases produced by ethanol may alter the spectrum of MHC-I restricted peptides presented on the hepatocyte cell surface and/or the expression levels of MHC-I. This may be a contributing factor to the autoimmune damage of the liver cell observed in long-term alcohol abuse (Chedid et al. 1993; Kachi et al. 1993).

Figure 3 summarizes some substrate proteins of the ubiquitin-proteasome pathway and the functions that may be perturbed as a result of ethanol treatment. The list of substrates discussed here is very limited and has focused primarily on those present on the ER membrane. A host of other proteins undergo regulated turnover via this pathway, including cyclins (Hershko 1997) and several protein kinase C isoforms (Lu et al. 1998; Lee et al. 1997). It is apparent that the proteasomal machinery is at a regulatory crossroad, controlling the levels of many key proteins that perform critical functions in the cell. The challenge remains to identify the molecular mechanism by which alcohol and its reactive metabolites interfere with this proteolytic pathway.

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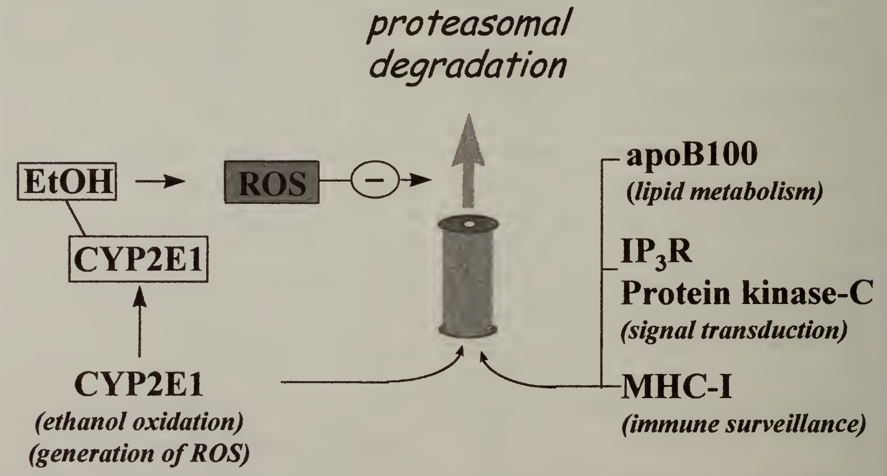

Figure 3. Inhibition by ethanol (EtOH) of the turnover of other substrate proteins in the liver that involve the ubiquitin-proteasome pathway. Inhibition of proteasomal peptidases by chronic EtOH treatment could affect a number of key proteins implicated in the pathogenesis of liver injury. Cytochrome P-450$_{2E1}$ (CYP2E1) is degraded via proteasomes. Binding of substrates (e.g., EtOH) stabilizes the protein and increases the generation of reactive oxygen species (ROS), which inhibits proteasomal activity. See the text for additional details on the other substrates. apoB100 = apolipoprotein B-100; IP$_3$R = inositol trisphosphate receptor; MHC-I = major histocompatibility complex class I.

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ETHANOL AND PROTEIN KINASE SIGNALING: PHYSIOLOGICAL RESPONSES

Chapter 4

Ethanol-Induced Translocation of Protein Kinases: A Possible Mechanism Underlying Ethanol-Induced Behaviors

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Key words: protein kinases; chronic AODE (effects of AOD [alcohol or other drug] use, abuse, and dependence); cell signaling; biological transport; Golgi apparatus; temporal context; biological inhibition; AOD sensitivity; histologic study

Numerous hormones, neurotransmitters, growth factors, and other signaling molecules alter the activity of adenylyl cyclase or phospholipase C. Cellular responses to 3′-5′-cyclic adenosine monophosphate (cAMP) and diacylglycerol (DAG), the second messengers generated by these enzymes, are mediated via the activation of cAMP-dependent protein kinase (PKA) and protein kinase C (PKC), respectively. Both PKA and PKC then regulate the activity of multiple cellular proteins by addition of a phosphate group to specific sites within the protein. Since both kinases are fairly ubiquitous, with only limited substrate specificity, it has been proposed that specificity is due to specific location of these kinases within the

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cell (Faux and Scott 1996; Mochly-Rosen and Gordon 1998). For example, Scott and colleagues have characterized a family of proteins called A kinase anchoring proteins (AKAPs) whose function appears to be to anchor the PKA holoenzyme near a substrate so that, on activation, the enzyme phosphorylates nearby substrates (Dell'Acqua and Scott 1997). A similar theme has been described for the PKC family by Mochly-Rosen and colleagues (Mochly-Rosen and Gordon 1998). Each PKC isozyme appears to bind to specific anchoring proteins, RICKs (receptors for inactive C-kinase), when in an inactive state. Upon activation, each isozyme translocates to a new subcellular location, where it binds to a second anchoring protein, RACK (receptor for activated C-kinase). Binding of each PKC isozyme to a corresponding RACK is necessary for its function. Therefore, it is apparent that subcellular localization is an extremely important and highly regulated determinant of kinase specificity, and anything that disrupts this localization would have detrimental effects on cellular function.

Our laboratory has documented that acute and chronic ethanol exposure produces remarkable translocation from one intracellular site to another of the catalytic subunit (Cα) of PKA and δ and εPKC in neural cells in culture. Ethanol-induced translocation of these protein kinases should result in altered regulation of numerous proteins and produce changes in gene expression as well as function of neurotransmitter receptors and ion channels, which may ultimately lead to ethanol-induced behaviors.

EFFECTS OF ETHANOL ON THE LOCALIZATION OF PKA Cα IN NG108-15 CELLS

We used a neural cell model and immunocytological techniques to examine the effects of ethanol exposure on localization of protein kinases. NG108-15 neuroblastoma x glioma hybrid cells were grown in culture in the presence or absence of ethanol for as long as 48 hours. We then examined the localization of one of the catalytic subunits of PKA, Cα, by staining fixed cells using an antibody specific for Cα. We found a dramatic difference in the localization of Cα in cells exposed to ethanol. In cultures exposed to 200 mM ethanol for 48 hours, 75 percent of the cells had Cα staining confined primarily to the nucleus (figure 1). In contrast, in control cultures, Cα staining was localized to the perinuclear Golgi area in ~80 percent of the cells. Localization of Cα to the Golgi in control cells was confirmed by double-labeling the cells with the Golgi-specific marker *N*-((4-(4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-yl)phenoxy)acetyl)sphingosine (BODIPY® TR ceramide) (see Dohrman et al. 1996).

The percentage of cells with primarily nuclear staining was dependent on ethanol concentration. Following 48 hours of ethanol exposure, significant translocation to the nucleus was detected at ethanol concentrations as low as 50 mM (figure 2). Longer durations of exposure to lower ethanol concentrations also produced significant accumulation of Cα in the nucleus; 63 ± 8 percent of cells exhibited nuclear staining following a 4-day exposure to 25 mM ethanol.

Ethanol-induced translocation of Cα to the nucleus was also time dependent. Although Cα could be observed in the cytoplasm as rapidly as 5 minutes after addition of ethanol to the culture media, significant accumulation of Cα in the nucleus required 6 hours of ethanol exposure (figure 3). This ethanol-induced translocation of Cα away from the Golgi and into the nucleus was maximal within 12 hours and persisted as long as ethanol was present. Sustained ethanol-induced Cα translocation is in contrast to the transient translocation induced by forskolin, an agent that activates PKA by increasing cAMP levels. In response to forskolin, Cα leaves the Golgi rapidly but returns after longer exposures (see figure 3). When ethanol is removed from the culture media, Cα returns to the Golgi with a time course similar to its initial accumulation in the nucleus; appearance in the Golgi area requires several hours and is maximal about 12 hours after the removal of ethanol (Dohrman et al. 1996).

EFFECTS OF ETHANOL ON THE LOCALIZATION OF δPKC AND εPKC IN NG108-15 CELLS

We also examined the effects of ethanol exposure on the localization of δ and εPKC, two isozymes of PKC known to up-regulate in response to chronic ethanol (Messing et al. 1991;

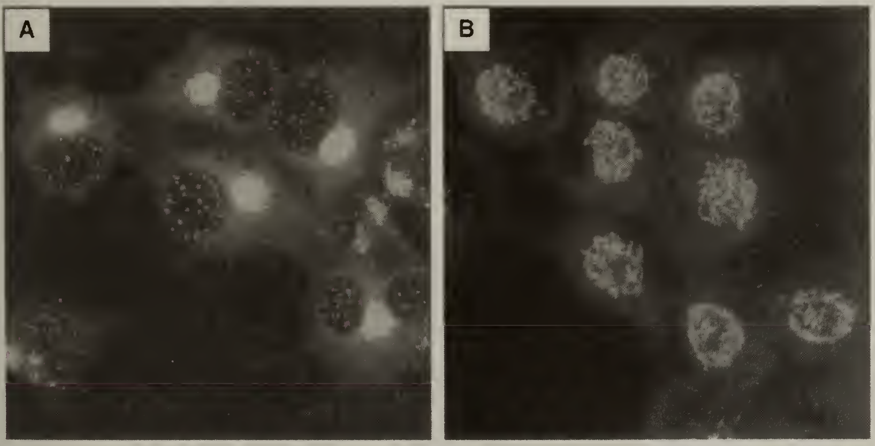

Figure 1. Ethanol-induced translocation of PKA catalytic subunit (Cα) in NG108-15 cells. (A) Cα is found primarily in the Golgi area in control cells. (B) After a 48-hour exposure to 200 mM ethanol, Cα is located predominantly in the nucleus. The images are 60x magnifications obtained with a Bio-Rad 1024 confocal microscope. Reprinted with permission from Dohrman, D.P.; Diamond, I.; and Gordon, A.S. Ethanol causes translocation of cAMP-dependent protein kinase catalytic subunit to the nucleus. *Proceedings of the National Academy of Sciences of the United States of America* 93:10217–10221, September 1996. Copyright (1996) National Academy of Sciences, U.S.A.

Coe et al. 1996*b*). Using NG108-15 cells, we found that ethanol induces a translocation of both δPKC and εPKC to new subcellular locations (figure 4). Similar to PKA Cα, δPKC was localized to the Golgi area in control cells. After incubation of cells for 48 hours in media containing 200 mM ethanol, δPKC translocated to the perinucleus and nucleus. This staining pattern was observed in more than 90 percent of ethanol-exposed cells, compared with less than 5 percent of control cells (figure 5).

Ethanol also induced translocation of εPKC. εPKC was localized to the perinuclear area in 95 percent of control cells (see figures 4 and 5). Following chronic ethanol exposure, εPKC staining was observed throughout the cytoplasm in more than 90 percent of cells. ε staining could also

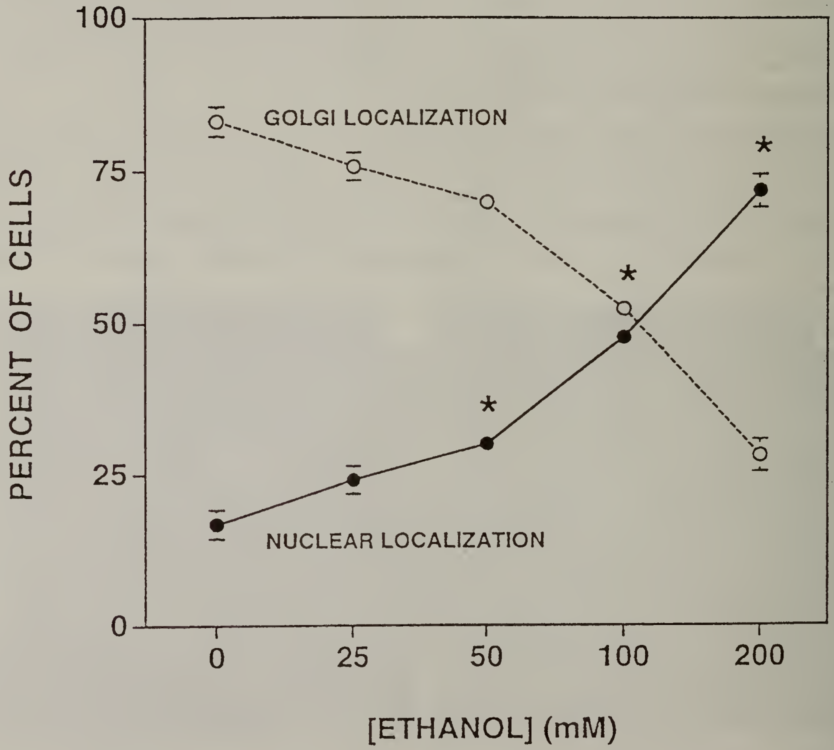

Figure 2. Translocation of Cα in NG108-15 cells as a function of ethanol concentration. Data points are the means ± SEM of four experiments. * $p < 0.05$ by analysis of variance with Scheffé post hoc comparison. Reprinted with permission from Dohrman, D.P.; Diamond, I.; and Gordon, A.S. Ethanol causes translocation of cAMP-dependent protein kinase catalytic subunit to the nucleus. *Proceedings of the National Academy of Sciences of the United States of America* 93:10217–10221, September 1996. Copyright (1996) National Academy of Sciences, U.S.A.

be detected in the nucleus, and perinuclear staining was still present, although decreased in intensity (Gordon et al. 1997).

We observed an important difference between ethanol-induced translocation of δ and εPKC. Translocation of δPKC to the nucleus and perinucleus was similar to that induced by the PKC activator, β-phorbol 12-myristate 13-acetate (β-PMA) (see figure 4). However, ethanol-induced translocation of εPKC was to a location different from that induced by β-PMA. Incubation of cells with β-PMA caused εPKC to translocate primarily to the nucleus, in contrast with ethanol-induced translocation to the cytoplasm (see figure 4).

Examination of the time course of ethanol-induced translocation of δPKC and εPKC revealed a different pattern from that of PKA Cα nuclear accumulation. Translocation of both δPKC (data not shown) and εPKC (figure 6) occurred rapidly, within minutes, and was maximal within 30 minutes. However, similar to PKA Cα, δPKC and εPKC remained in their altered subcellular locations as long as ethanol was present. Altered localization of δ and εPKC in response to brief ethanol

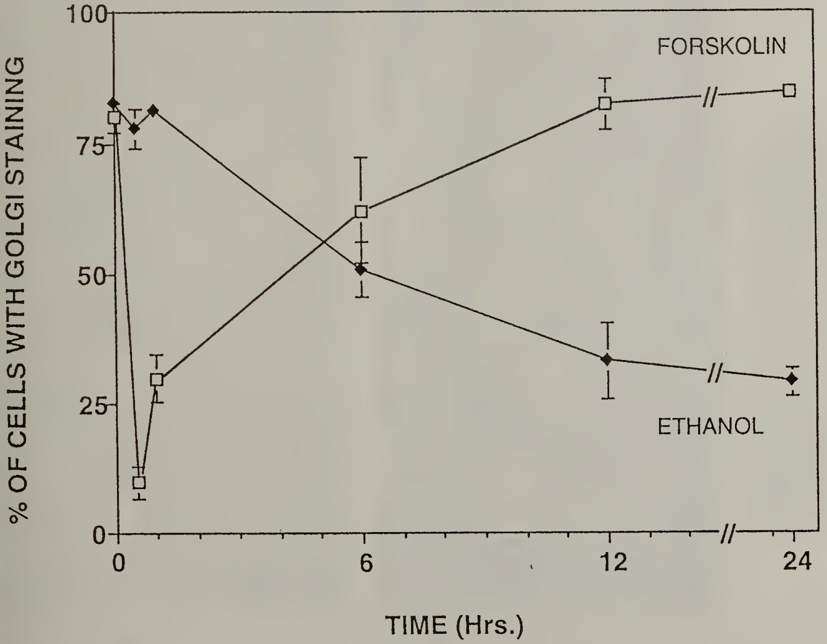

Figure 3. Time course of translocation of Cα in NG108-15 cells exposed to 200 mM ethanol or 1 mM forskolin. Data points are the means ± SEM of three experiments. Reprinted with permission from Dohrman, D.P.; Diamond, I.; and Gordon, A.S. Ethanol causes translocation of cAMP-dependent protein kinase catalytic subunit to the nucleus. *Proceedings of the National Academy of Sciences of the United States of America* 93:10217–10221, September 1996. Copyright (1996) National Academy of Sciences, U.S.A.

exposure was most likely due to translocation of existing kinase and not new synthesis, since Western blot analysis revealed no changes in protein synthesis at these short time points (Gordon et al. 1997). However, chronic ethanol exposure does result in increased levels of both δ and εPKC (Coe et al. 1996*b*). In contrast, chronic ethanol exposure does not alter levels of PKA Cα (Dohrman et al. 1996).

IMPLICATIONS FOR THE BEHAVIORAL EFFECTS OF ETHANOL

PKA and PKC modulate the activity of multiple cellular proteins, including

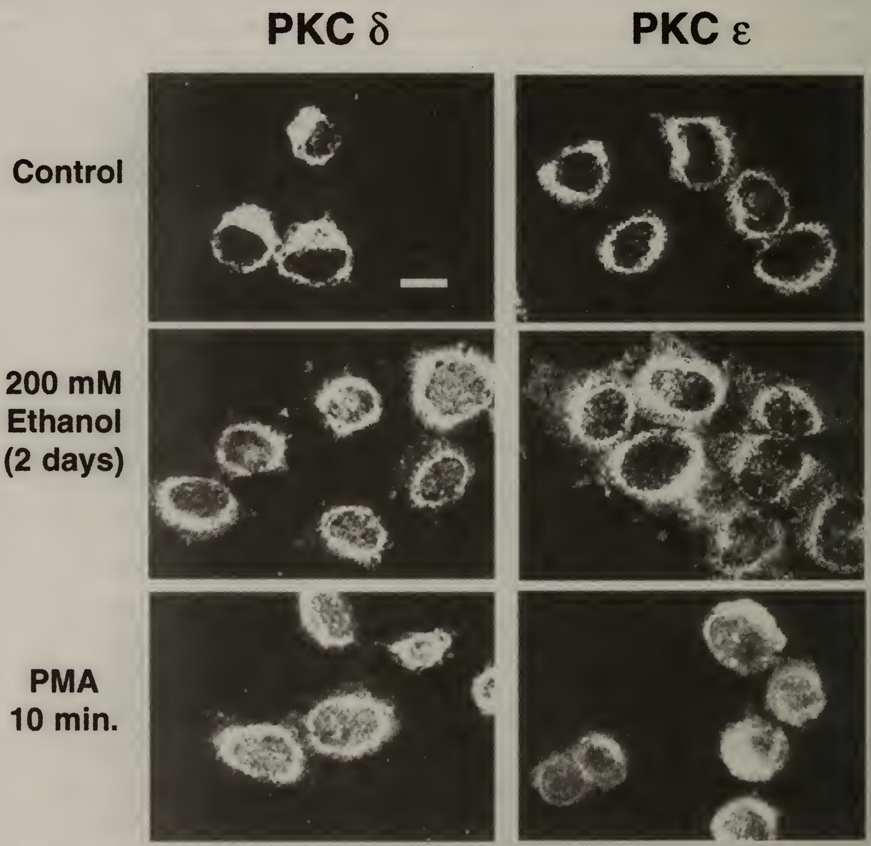

Figure 4. Ethanol induces reversible translocation of δPKC and εPKC. NG108-15 cells were incubated with 200 mM ethanol for 2 days or with 100 nM β-phorbol 12-myristate 13-acetate (β-PMA) for 10 minutes. Cells were fixed and stained for δ and εPKC using antibodies specific for each isozyme. Images were obtained with a Bio-Rad 1024 confocal microscope. *Bar*, 25 μm. Reprinted with permission from Gordon, A.S.; Yao, L.; Wu, Z.-L.; Coe, I.R.; and Diamond, I. Ethanol alters the subcellular localization of δ- and ε protein kinase C in NG108-15 cells. *Molecular Pharmacology* 52(4):554–559, October 1997.

nuclear, cytoplasmic, and membrane proteins. For example, both PKA and PKC phosphorylate and modulate *N*-methyl-D-aspartate (NMDA) receptors (Raymond et al. 1993; Leonard and Hell 1997; Tingley et al. 1997). Since there is little substrate specificity for these kinases, phosphorylation of the appropriate substrate(s) in response to signaling molecules is thought to be determined by subcellular localization within the cell (Faux and Scott 1996; Mochly-Rosen and Gordon 1998). The importance of localization of kinases in cellular function is supported by the observation that inhibitors of translocation block kinase function. For example, using a peptide that blocks the binding of PKA regulatory subunit to AKAPs, Catterall and colleagues demonstrated a loss of potentiation of L-type cal-

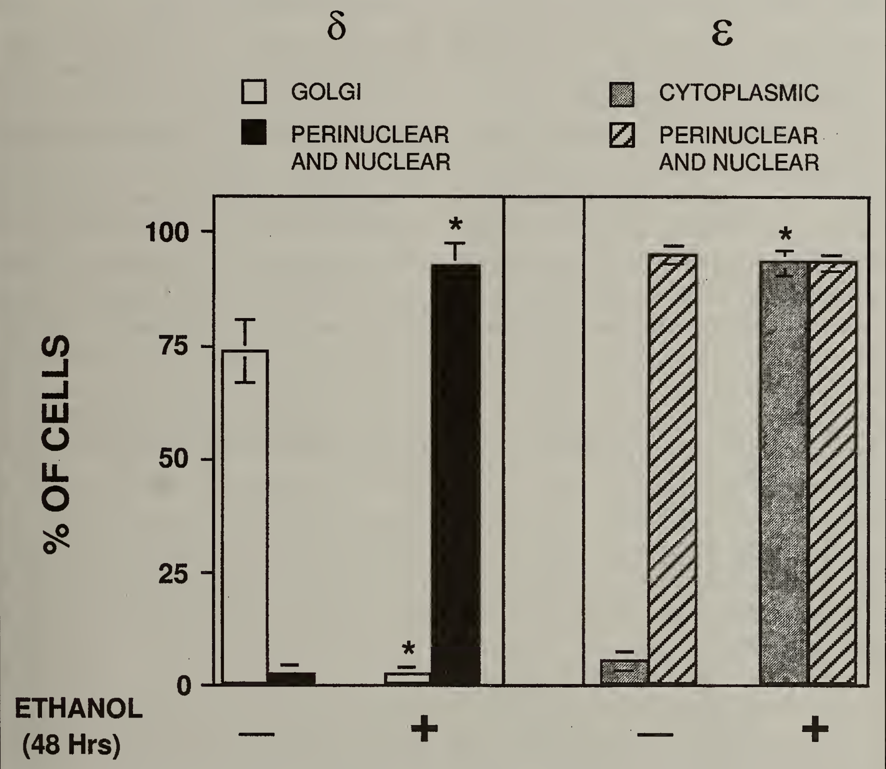

Figure 5. Quantitation of ethanol-induced translocation of δPKC and εPKC. NG108-15 cells were incubated in the absence or presence of 200 mM ethanol for 48 hours and then fixed and stained for δ and εPKC. Data shown are the means ± SEM of five experiments. * $p < 0.05$ compared with localization in control cells (two-way analysis of variance and Newman-Keuls test). Reprinted with permission from Gordon, A.S.; Yao, L.; Wu, Z.-L.; Coe, I.R.; and Diamond, I. Ethanol alters the subcellular localization of δ- and ε protein kinase C in NG108-15 cells. *Molecular Pharmacology* 52(4):554–559, October 1997.

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cium channels by endogenous PKA, suggesting that close physical proximity between the channel and PKA is critical for potentiation (Johnson et al. 1994). Scott's laboratory has also demonstrated that targeting of PKA to the plasma membrane regulates cAMP-responsive calcium currents (Fraser et al. 1998). In addition, Rosenmund and colleagues (1994) demostrated that perfusion of cultured hippocampal neurons with the anchoring inhibitor peptide caused a decrease in α-amino-3-hydroxy-5-methyl isoxazole-4-propionic acid (AMPA)/kainate-responsive currents. The localization of PKA also determines the rate and magnitude of cAMP signaling to the nucleus (Cassano et al. 1996; Feliciello et al. 1997). In a mutated PC12 cell line, in which PKA is primarily cytosolic instead of membrane-associated as in the parental cell line, PKA does not accumulate in the nucleus nor transcribe proteins with cAMP-dependent promoters in response to cAMP.

Similar to PKA, when PKC is activated it translocates to a specific location within the cell, where it binds to an isozyme-specific RACK and phosphorylates nearby substrates (Mochly-Rosen 1995; Mochly-Rosen and Gordon 1998). Peptides that block the binding of specific PKC isozymes to corresponding RACKs alter PKC function (Ron and Mochly-Rosen 1994; Ron et al. 1995; Johnson et al. 1996). Interestingly, a scaffolding protein, which anchors both PKA and PKC near substrates, has been described by Klauck and colleagues (1996). These studies demonstrate that localization of PKA and PKC within the cell is tightly regulated and important for determining substrate

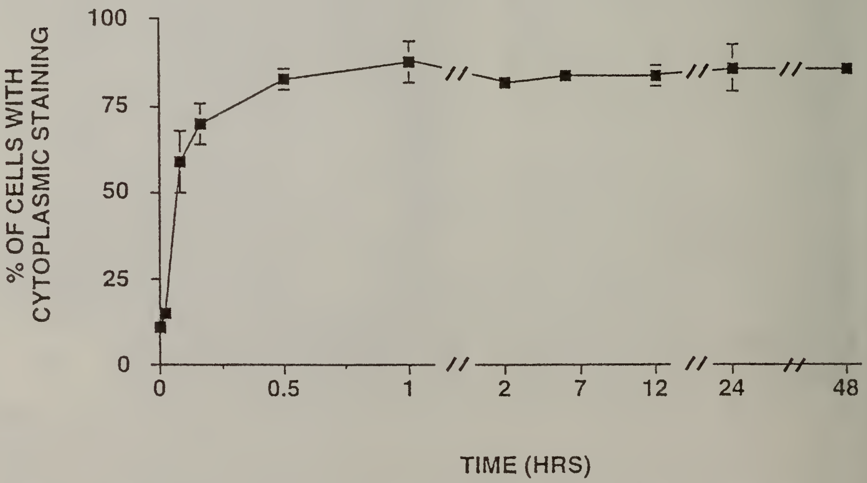

Figure 6. Ethanol (200 mM) causes a time-dependent translocation of εPKC. Data points are means ± SEM for three experiments. Reprinted with permission from Gordon, A.S.; Yao, L.; Wu, Z.-L.; Coe, I.R.; and Diamond, I. Ethanol alters the subcellular localization of δ- and ε protein kinase C in NG108-15 cells. *Molecular Pharmacology* 52(4):554–559, October 1997.

specificity and cellular function. Therefore, ethanol-induced alterations in kinase localization should have dramatic effects on phosphorylation and function of specific substrates.

Kinases also appear to determine the sensitivity of receptors to modulation by ethanol. For example, the ethanol sensitivity of the gamma-aminobutyric acid type A ($GABA_A$) receptor and the NMDA receptor appears to be regulated by PKC phosphorylation (Wafford et al. 1991; Snell et al. 1994; Macdonald 1995). Indeed, there is diminished ethanol sedation in a mouse that lacks the γPKC isozyme (Harris et al. 1995). Ethanol inhibition of calcium channels has also been shown to be regulated by PKA (Solem et al. 1997). We have shown that the ethanol sensitivity of a transporter of the neuromodulator adenosine, which regulates the effects of ethanol on cAMP levels in a neural cell line, appears to be dependent on PKA activity (Coe et al. 1996*a*). We also found that tolerance to the acute effects of ethanol on adenosine transport develops following chronic exposure to ethanol and proposed that tolerance is due to decreased phosphorylation of the transporter mediated by translocation of PKA away from the transporter (Coe et al. 1996*a*; Dohrman et al. 1996).

PKA may also be involved in the addictive properties of ethanol. In vivo ethanol exposure produced alterations in the phosphorylation of cAMP-response element binding protein (CREB) in the striatum of rats (Yang et al. 1998), an area of the brain thought to be involved in the reinforcing effects of ethanol. CREB is a transcription factor that regulates the expression of multiple genes and is activated when phosphorylated by PKA after the catalytic subunit translocates to the nucleus. Acute ethanol exposure resulted in increased CREB phosphorylation. However, in animals that were chronically exposed to ethanol and then rechallenged with acute ethanol, CREB phosphorylation was markedly attenuated.

Other data have also suggested a role for PKA in ethanol addiction. Nestler and colleagues proposed that PKA mediates changes in the nucleus accumbens as a common, underlying mechanism of addiction to ethanol and other drugs of abuse (Self and Nestler 1995). In this model, ethanol causes the release of dopamine in the nucleus accumbens, which has been related to reinforcement of drug reward. Dopamine receptors are coupled to G proteins, which regulate adenylyl cyclase and cAMP production. The inhibition or activation of PKA has been proposed to enhance or oppose reinforcement, respectively. Moreover, translocation of the PKA catalytic subunit to the nucleus in these neurons has been suggested to alter gene expression and thus produce craving. Indeed, there is a great deal of evidence to support a role of PKA in addiction. For example, agents that alter PKA activity also alter drug self-administration when injected into the nucleus accumbens (for a review, see Nestler and Aghajanian 1997). Recent work in *Drosophila* (Moore et al. 1998) also implicates PKA in mediating ethanol's behavioral effects. Genetic alterations in the cAMP pathway and pharmacological alteration of PKA activity modulate the ethanol sensitivity of fruit flies (see chapter 13 in this monograph).

In conclusion, there is accumulating evidence for the involvement of PKA- and PKC-mediated phosphorylation in mediating some of the behavioral responses to acute and chronic ethanol exposure. Ethanol alters PKA and PKC signaling pathways and this may, in turn, alter the ethanol sensitivity of other proteins. Ethanol-induced alterations in subcellular localization of PKA and PKC may play an important role in determining which proteins are affected by ethanol and also could regulate gene expression by increasing the phosphorylation of substrates in the nucleus. Such increases in gene expression may lead to changes in the brain that underlie alcoholism and characteristic behaviors such as tolerance, dependence, and craving.

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Chapter 5

Ethanol-Mediated Protection of Cardiac Myocytes From Ischemic Injury

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Key words: ethanol; protective factors; myocardial ischemia or hypoxia; cardiomyocyte; chronic AODE (effects of AOD [alcohol or other drug] use, abuse, and dependence); alcohol cardiomyopathy; heavy AOD use; moderate AOD use; mortality

The effects of ethanol on the cardiovascular system have long been of interest to public health officials, epidemiologists, physicians, and scientists. Although certain genetic backgrounds or preexisting cardiac disease may leave individuals more vulnerable to ethanol toxicity, in general the detrimental effects of ethanol on cardiac contractile function are linked to heavy consumption and the cardioprotective effects to moderate consumption (Klatsky et al. 1992; Hanna et al. 1997). According to the *Provisional Table on the Nutrient Content of Beverages* (U.S. Department of Agriculture, Human Nutrition Information Service), 12 oz of beer, 5 oz of wine, and 1½ oz of 80-proof distilled spirits each contain 8 g of alcohol and are considered "drink equivalents." The Dietary Guidelines Advisory Committee (1995) defined moderate alcohol intake as one drink or less per day for women and two drinks or less per day for men. Heavy alcohol consumption has not been as precisely defined, but the pathological effects of abuse are well described and cardiomyopathy is a common diagnosis in alcoholics (Rubin and Urbano-Marquez 1994).

Alcoholic heart muscle disease (AHMD) is said to exist in humans when the sole causative agent is excessive

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ethanol consumption with intake of greater than 80 g/day for 10 years or more or a cumulative lifetime ethanol intake exceeding 250 kg (Richardson and Wodak 1983; Richardson et al. 1986). The effect of high ethanol concentration to depress myocardial contractility acutely has been studied best in isolated rat heart models. For example, perfusion with ethanol (0.75–3.0 vol %; 128–512 mM) causes a concentration-dependent decrease in left ventricular (LV) developed pressure, ultimately leading to complete loss of developed pressure and abolition of electrical activity with infusion of 6 vol % (1,024 mM) ethanol (Schulman et al. 1991). Acute reductions in contractility are not accompanied by changes in high-energy phosphates, inorganic phosphate, or pH as measured by ^{31}P nuclear magnetic resonance spectroscopy and are rapidly reversed by perfusion with ethanol-free solution. Concurrent decreases in amplitude of intracellular Ca^{2+} transients (Kojima et al. 1993) and reversal of reduced contractility by increases in perfusate calcium concentration (Schulman et al. 1991) support the hypothesis that ethanol acutely alters myocardial Ca^{2+} handling and excitation-contraction coupling mechanisms.

Heavy alcohol consumption alters myocardial contractility in chronic rat models as well. Hearts from Sprague-Dawley rats receiving 36 percent ethanol (vol/vol) in their drinking water for 7 months (mean blood ethanol concentration at time of sacrifice = 144 ± 29 mg/dL) show a reduction in LV developed pressure, limited ability to withstand rapid pacing, and faster rates of contraction and relaxation (Figueredo et al. 1998). Chronic ethanol exposure in this model does not affect Ca^{2+} handling, protein levels of SERCA2a and phospholamban, or myosin heavy chain isoform content, suggesting abnormalities of excitation-contraction coupling at the level of the myofilament that do not involve myosin heavy chain isoform shifts. In human AHMD, imaging of the left ventricle reveals frank dilatation and depressed contractility (Richardson and Wodak 1983; Richardson et al. 1986). Abnormalities in biopsy and pathologic specimens from AHMD patients include disruption of myofibrillar architecture, mitochondrial degeneration, widening of gap junctions, dilation of the sarcoplasmic reticulum and transverse tubule system, fibrosis, and lipopigmentation (Ferrans et al. 1965; Hibbs et al. 1965). Patients with alcoholic cardiomyopathy have a mortality rate of 50 percent over 4 years with continued consumption (Demakis et al. 1974). Surprisingly, AHMD is largely reversible; abstention from further alcohol use leads to dramatic improvement in mortality and LV ejection fraction.

In contrast to the toxic effects of heavy ethanol exposure on myocardial structure and function, moderate alcohol consumption protects against coronary heart disease (CHD). In the Framingham study, CHD mortality over 24 years of followup was examined in relation to alcohol intake in men and women (Gordon and Kannel 1983; Friedman and Kimball 1986). Men who drank alcohol in moderate amounts had a lower risk of CHD than abstainers, with greatest protection against myocardial infarction.

The lowest risk of coronary mortality for women in Framingham was observed at alcohol consumption of one-half to one drink per day, with greatest protection against angina pectoris. Because it is unlikely that a randomized clinical trial of alcohol and CHD end points will ever be done, efforts to identify the mediators of ethanol-induced cardiac protection in humans focus on detailed statistical modeling of observational data. For example, in both the Lipid Research Clinics Follow-up Study (Criqui et al. 1987) and the Honolulu Heart Study (Langer et al. 1992), about 50 percent of the protective effect of alcohol was attributed to increased high-density lipoprotein (HDL) cholesterol levels. Moderate alcohol consumption may also protect against CHD by decreasing platelet aggregation (Renaud et al. 1992) and by increasing plasma concentration of endogenous tissue-type plasminogen activator (Ridker et al. 1994).

In addition to halting the progression of atherosclerosis and reducing the potential for thrombosis, moderate ethanol exposure may induce protection through direct effects on the heart. Hartley guinea pigs receiving 2.5–20 vol % ethanol in their drinking water for 3–12 weeks do not differ from age-matched control animals in LV developed pressure or coronary flow (Miyamae et al. 1997). However, hearts from control and ethanol-treated guinea pigs differ significantly in their ability to withstand the stress of myocardial ischemia-reperfusion injury. Following 45 minutes of no-flow ischemia, isolated hearts from animals treated with ethanol show improved recovery of LV developed pressure when compared with control hearts (52 percent vs. 31 percent of pre-ischemic values; $p < 0.05$). Ethanol-preconditioned hearts also release significantly less creatine kinase following global ischemia and reperfusion when compared with control hearts (159 ± 25 vs. 356 ± 26 units/mL/g dry weight; $p < 0.05$), suggesting that chronic moderate exposure to ethanol improves functional recovery and decreases myocyte injury (Miyamae et al. 1997).

Ischemic preconditioning, a protective mechanism in which the heart is exposed to brief sublethal ischemia that attenuates cellular damage from a subsequent prolonged period of ischemia, has been studied extensively in a variety of isolated heart models. Although the mediators of preconditioning have not been definitively identified, there is substantial experimental evidence that one of the major intracellular signal transduction pathways underlying cardiac protection involves activation of protein kinase C (PKC) (Simkhovich et al. 1998). For example, activation of PKC by transient ischemia or by α_1 adrenergic receptor agonists in an isolated rat heart model induces protection during sustained global ischemia and reperfusion that is inhibited by PKC antagonists (Mitchell et al. 1995). Ethanol-induced protection of cardiac myocytes from ischemic injury may also be mediated by PKC activation.

In collaboration with Vincent Figueredo, we recently reported a guinea pig model of chronic moderate ethanol consumption in which cardiac protection was abolished by PKC inhibition with chelerythrine (Miyamae et al. 1998). In this study, Hartley guinea pigs were fed a nutritionally supplemented liquid diet containing 15 percent or 0 percent ethanol-derived calories for 8 weeks (figure 1). Hearts from control and treated animals were isolated and perfused via the Langendorff method. After a 20-minute equilibration period, hearts were subjected to 45 minutes of no-flow ischemia and 48 minutes of reperfusion. After reperfusion, hemodynamic measurements were repeated every 6 minutes for 48 minutes. Additional experiments were performed with chelerythrine chloride, a potent selective inhibitor of the catalytic domain of PKC (Herbert et al. 1990), added to the perfusate 10 minutes prior to ischemia (see figure 1).

In this model serum ethanol levels were 10 ± 2 mg/dL (2 mM) after 8 weeks of feeding with a liquid diet containing 15 percent ethanol-derived calories. Body weight, dry heart weight to body weight ratio, baseline LV developed pressure, coronary flow, and perfusion pressure were the same in control and ethanol-fed animals. However, hearts from control and ethanol-treated animals differed significantly in resistance to ischemia-reperfusion injury. LV developed pressure recovered to 42 percent of pre-ischemic levels in hearts from ethanol-fed animals compared with 22 percent in control hearts ($p < 0.05$). Increased LV end-diastolic pressure, an index of myocyte contracture and injury, was lower during reperfusion in hearts from ethanol-fed animals compared with control hearts (350 percent vs. 580 percent; $p < 0.05$). Creatine kinase release was lower from hearts of ethanol-fed animals compared with control hearts (260 ± 40 vs. 469 ± 74 units/mL/g dry weight; $p < 0.05$). The PKC inhibitor chelerythrine abolished differences in resistance to ischemia-reperfusion injury, supporting a critical role for PKC activation in the signal transduction pathways underlying ethanol-induced cardiac protection

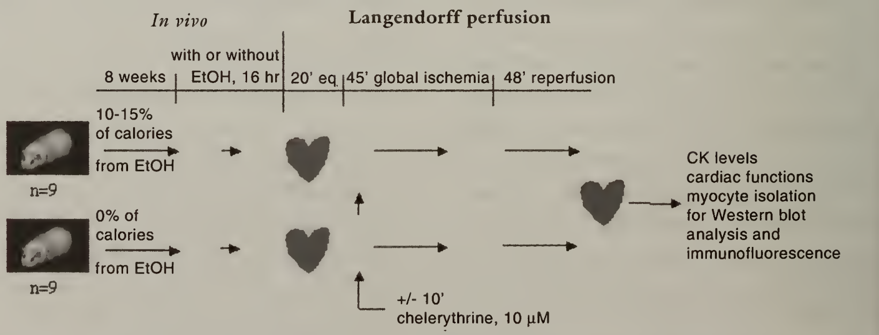

Figure 1. Schematic presentation of the experimental protocol used to determine the cardioprotective effect of chronic exposure to ethanol (EtOH) in vivo, using a guinea pig model of moderate ethanol consumption. Experimental data are described in the text and in Miyamae et al. 1998. eq. = equilibration; CK = creatine kinase.

(Miyamae et al. 1998). Importantly, protection obtained by chronic moderate ethanol exposure in vivo was unchanged by termination of ethanol exposure 16 hours prior to heart isolation compared with termination immediately prior to isolation. These data suggest a long-term and sustained cardioprotective effect due to ethanol exposure in vivo.

Protein kinase C is actually a family of closely related isozymes whose kinase activities depend to varying extents on the presence of phospholipid, diacylglycerol, and calcium (Nishizuka 1992). PKC isozymes also differ in subcellular localization before and after activation (Mochly-Rosen 1995). For example, in cultured neonatal rat cardiac myocytes β_IPKC localizes to perinuclear structures in control cells but to the nuclei of myocytes stimulated with phorbol ester or norepinephrine (Disatnik et al. 1994). These differences in sensitivity to biological activators and in subcellular localization suggest that particular PKC isozymes play distinct roles in cardiac myocyte function. At least six different PKC isozymes have been identified in rat cardiac myocytes (Disatnik et al. 1994), and at least ten isozymes are expressed in the rabbit heart (Ping et al. 1997). In a conscious rabbit heart model, ischemic preconditioning resulting from one to six cycles of 4–5 minute coronary occlusion produces no change in either total PKC activity or in the distribution of total PKC activity between the cell soluble and cell particulate fractions (Ping et al. 1997). However, ischemic preconditioning does cause significant translocation of εPKC and ηPKC isoforms from the cytosolic to the particulate fraction in the same model using quantitative immunoblotting techniques. These results support the conclusions that measurements of total PKC activity are not sufficiently sensitive to detect the involvement of PKC in preconditioning and that εPKC and/or ηPKC play important roles in ischemic preconditioning in the conscious rabbit heart model (Ping et al. 1997).

In the guinea pig model of chronic moderate ethanol consumption described earlier, we found that activation of εPKC correlated with ethanol-induced cardiac protection (Miyamae et al. 1998). As shown in figure 1, Hartley guinea pigs were fed with 10 percent or 0 percent ethanol and solid food ad libitum for 8 weeks. Cardiac myocytes were isolated by perfusing hearts from control and ethanol-treated animals with Krebs-Henseleit buffer containing collagenase B. Western blot analysis was used to determine the subcellular distribution of αPKC, δPKC, and εPKC in control and ethanol-treated myocytes. Immunofluorescence staining was used to corroborate immunoblotting data and to characterize more precisely the localization of εPKC. We found by Western analysis that subcellular distribution of αPKC and δPKC did not differ in cardiac myocytes from control and ethanol-fed animals. In contrast, the ratio of particulate to cytosolic εPKC was greater in myocytes from ethanol-fed compared with control animals (2.7 ± 0.1 vs. 1.5 ± 0.4; $p < 0.05$). Immunofluorescence staining also failed to demonstrate ethanol-induced translocation of αPKC and δPKC. However, the percentage of myocytes exhibiting activated εPKC in a cross-striated pattern of fluorescence was greater in hearts from ethanol-fed animals compared with controls (61 ± 3 percent vs. 22 ± 5 percent; $p < 0.05$). These data suggest that εPKC is significantly translocated or redistributed in cardiac myocytes following regular moderate ethanol consumption (Miyamae et al. 1998).

The argument that εPKC activation and translocation are critical components of ethanol-mediated preconditioning would be strengthened by demonstration that cardiac protection is diminished or abolished by PKC isoform-selective inhibition. We recently cloned two proteins termed "RACKs" (receptors for activated C-kinase) that bind activated PKC through a site distinct from the substrate binding site (Ron et al. 1994; Csukai et al. 1997). Our current model of the relationship between PKC and RACKs is that PKC binding to RACKs occurs after cell stimulation leading to the anchoring of each active isozyme close to its physiological substrates (Mochly-Rosen and Gordon 1998). This model predicts that peptides that compete for binding of PKC to RACKs would act as isozyme-selective inhibitors of PKC translocation and function (Mochly-Rosen 1995; Souroujon and Mochly-Rosen 1998). Such isozyme-selective peptide inhibitors have been developed and used successfully in a variety of cell systems to determine the functional significance of particular PKC isozymes. For example, a peptide called βC2-4 that corresponds to amino acids 218–226 within the C2 region of βPKC binds to RACK1 in vitro and interferes with binding of β_{II}PKC to RACK1 in vivo (Ron et al. 1995). Furthermore, βC2-4 peptide microinjected into *Xenopus* oocytes significantly delays insulin-induced oocyte maturation, a process previously shown to correlate with translocation of βPKC (Ron and Mochly-Rosen 1994; Ron et al. 1995). As a second example, a peptide called εV1-2 that corresponds to amino acids 14–21 within the V1 region of εPKC selectively inhibits in neonatal rat cardiac myocytes both the translocation of εPKC and the negative chronotropic effect of 4β-phorbol 12-myristate 13-acetate (PMA), a process previously shown to correlate with translocation of εPKC (Johnson and Mochly-Rosen 1995; Johnson et al. 1996). More recently, εV1-2 peptide has been used successfully to investigate the role of εPKC activation and translocation in glucose-induced insulin release by pancreatic beta-cells (Yedovitzky et al. 1997) and in tumor necrosis factor alpha (TNFα)–induced apoptosis in U937 histiocytic lymphoma cells (Mayne and Murray 1998).

We have reported a neonatal rat cardiac myocyte culture model of hypoxic preconditioning in which cardiac protection was abolished by εPKC inhibition with εV1-2 peptide (Gray et al. 1997). In that study, cardiac myocytes from day-old Sprague-Dawley rats were cultured and preconditioned by brief transfers into and out of a Plexiglas chamber containing less than 0.5 percent O_2. Control and preconditioned myocytes were then subjected to a prolonged hypoxic incubation followed by assay of myocyte viability. In this model, preconditioned myocytes were more resistant to prolonged hypoxia compared with control cells (65 ± 3 percent vs. 34 ± 2 percent viable cells; $p < 0.05$). Direct activation of PKC with 10 nM PMA produced robust translocation of εPKC and mimicked the protective effect of hypoxic preconditioning. Hypoxic preconditioning produced a twofold increase in the percentage of myocytes exhibiting activated εPKC in a cross-striated pattern of immunofluorescence staining compared with control cells (49.9 ± 2.8 percent vs. 23.6 ± 2.6 percent, $p < 0.05$). Introduction of εV1-2 peptide antagonist into cardiac myocytes selectively inhibited translocation of εPKC as determined by Western blot analysis and abolished both hypoxia-induced and PMA-induced cardiac protection during subsequent prolonged hypoxia. Taken together, these data support the hypothesis that εPKC activation and translocation are necessary for the protective effect of hypoxic preconditioning in this cardiac myocyte culture model (Gray et al. 1997). Studies with a novel εPKC-selective agonist are under way in our laboratory, and preliminary data suggest that εPKC activation and translocation are sufficient to reduce cardiac death dramatically in this model (Dorn et al. 1999).

Our efforts to determine whether εPKC activation and translocation are necessary components of ethanol-mediated cardiac preconditioning are currently focused on the use of PKC isozyme-selective inhibitors in an isolated adult rat cardiac myocyte model of ischemia. In this model hearts are rapidly excised, cannulated via the aorta, and perfused with buffer containing collagenase B. Ventricular myocytes are isolated by maceration of the collagenase-digested sample, removal of connective tissue by filtration through stainless steel mesh, and centrifugation of the resultant cellular suspension. Myocytes can be further separated from nonmyocytes by passage of the cell pellet through a Ficoll-400 gradient (Miyamae et al. 1998). As shown in figure 2, cardiac ischemia is simulated by transfer of freshly isolated myocytes to microcentrifuge tubes, low-speed centrifugation, removal of supernatant, and layering of mineral oil or microballoons over the packed myocytes to create an airtight environment (Vander Heide et al. 1990). Cell samples can then be drawn from the pellet at given time intervals and assayed for viability. Typically, myocyte viability is determined by a membrane fragility test using hypotonic (85 mOsmol) 1 percent trypan blue solution. Cells that are unable to exclude the trypan blue dye are considered nonviable due to membrane failure, and the percentage of nonviable cells can be plotted over time (Vander Heide et al. 1990). This in vitro model of cardiac ischemia has proved to be a very useful approach to identification of potential cellular mediators of preconditioning because it permits direct comparison of control and acutely treated myocytes from the same cell isolation (Armstrong et al. 1997; Critz et al. 1997), as well as comparison of myocytes isolated from the hearts of control and chronically treated animals.

Recent experiments performed in our laboratory suggest that we have identified an isolated adult rat cardiac myocyte model for ethanol-mediated preconditioning and that εPKC activation and translocation are necessary components of ethanol-induced protection (Chen et al. 1999). Immediately after isolation, cardiac myocytes were treated with 50 mM ethanol for 10 minutes. Control and treated myocytes were pelleted by low-speed centrifugation and washed twice with ethanol-free incubation buffer. For hypoxic treatment, cells were transferred to microcentrifuge tubes, washed twice with degassed incubation buffer, and pelleted by low-speed centrifugation. The supernatant was removed, and microballoons were layered over the packed myocytes to create an airtight environment (see figure 2). Cell samples were drawn from pellets at given time intervals, and the percentage of nonviable myocytes (i.e., unable to exclude trypan blue dye) was plotted over time. We found no significant change in myocyte viability after 3 hours of incubation under normoxic conditions. In contrast, the percentage of nonviable control myocytes was doubled by 3 hours of incubation under hypoxic conditions. Furthermore, pretreatment with 50 mM ethanol for 10 minutes preconditioned cardiac myocytes for the stress of in vitro ischemia, doubling the percentage of viable myocytes present after 3 hours of incubation under hypoxic conditions.

Three lines of evidence support a critical role for εPKC activation and translocation in ethanol-mediated preconditioning in this model. First, the presence of the general PKC inhibitor chelerythrine chloride during ethanol

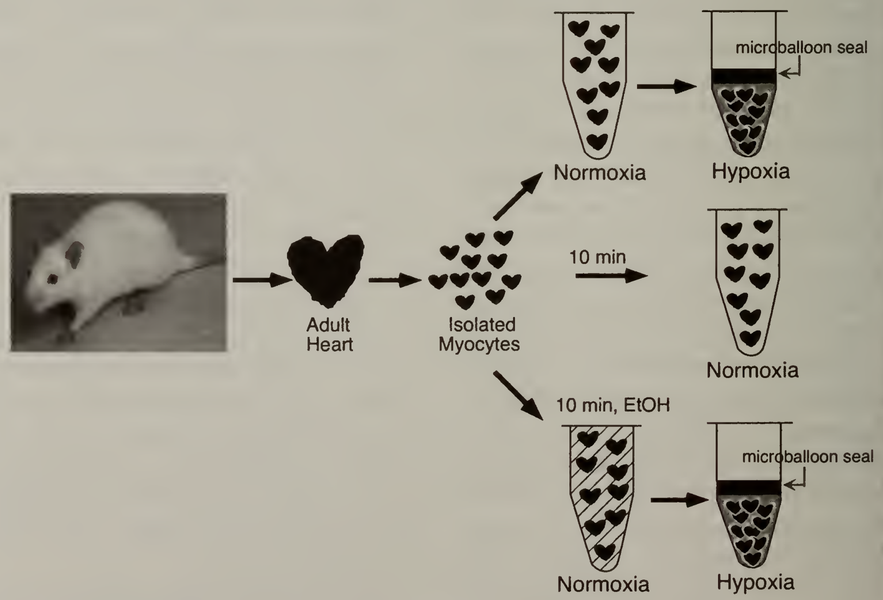

Figure 2. Schematic presentation of the experimental protocol used to determine the cardioprotective effect of acute exposure to ethanol (EtOH) in vitro, using an isolated adult rat cardiac myocyte model.

preconditioning of myocytes abolishes ethanol-induced protection during subsequent hypoxic incubation. Second, treatment of cardiac myocytes with 50 mM ethanol doubles the amount of εPKC that translocates from the cell soluble to the cell particulate fraction compared with control myocytes in Western blot assays of PKC activation. Finally, by conjugating εV1-2 peptide to Antennapedia-derived carrier peptide, a process that enhances entry into eukaryotic cells (Langer et al. 1992; Derossi et al. 1994), we have demonstrated rapid delivery of biologically active concentrations of this εPKC-selective inhibitor into cultured neonatal rat cardiac myocytes (Dorn et al. 1999) and into isolated adult rabbit cardiac myocytes (Liu et al. 1998). Introduction of εV1-2 peptide during ethanol preconditioning of isolated rat cardiac myocytes selectively inhibits ethanol-induced protection by more than 75 percent during subsequent hypoxic incubation.

In summary, moderate ethanol consumption by humans protects against CHD by inhibition of atherosclerosis and thrombosis and probably by direct effects on cardiac myocytes. Preconditioning of cardiac myocytes following moderate ethanol exposure has been studied most thoroughly in rodent heart models and involves PKC, particularly activation and translocation of the εPKC isozyme. Acute exposure of isolated cardiac myocytes to ethanol activates εPKC and protects cells during simulated ischemia in vitro. Importantly, chronic exposure of hearts to ethanol activates εPKC and induces sustained protection against ischemic injury in vivo. General public health recommendations regarding the cardiovascular benefits of moderate ethanol consumption must be tempered by consideration of co-existing medical and social conditions that may be worsened by alcohol. However, increased understanding of ethanol-mediated cardiac preconditioning will allow more insightful guidance of individual patients who wish to drink in moderation. Furthermore, identification of the cellular and molecular mediators of ethanol preconditioning in cardiac myocytes may result in development of therapeutic agents that maximize protection of heart tissue against ischemic injury while circumventing the detrimental effects of drinking.

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Chapter 6

Ethanol and IRS-1 Protein in the Liver and Brain

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In addition to direct toxic effects of ethanol and its metabolites on the liver, acute and chronic ethanol exposure impairs hepatocyte DNA synthesis in vitro (Carter and Wands 1985, 1988) and the ability of the liver to regenerate following partial hepatectomy (Wands et al. 1979; Diehl et al. 1990, 1992). The precise molecular mechanisms by which ethanol inhibits hepatocyte proliferation are poorly understood. Liver regeneration is regulated by several growth factors, of which tumor necrosis factor alpha (TNFα), epidermal growth factor (EGF), transforming growth factor alpha, interleukin-6, hepatocyte growth factor (HGF), and insulin are believed to be the most important ligands (Pistoi and Morello 1996; Michalopoulos and DeFrances 1997). Previous studies suggest that ethanol

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interferes with signal transduction cascades induced by HGF (Saso et al. 1996), EGF (Higashi and Hoek 1991; Zhang et al. 1996; Saso et al. 1997), and TNFα (Akerman et al. 1993) and with [Ca^{2+}]-mediated signals in hepatocytes (Zhang and Farrel 1997).

Insulin is known to promote hepatocyte proliferation in the context of other growth factors (Michalopoulos and DeFrances 1997) and is absolutely required for normal liver regeneration to occur (Bucher and Swaffield 1975; Starzl et al. 1976, 1978, Johnston 1986; Chin 1995; Knopp et al. 1997). Binding of insulin and insulin-like growth factor I (IGF-I) to their specific cell surface receptors leads to autophosphorylation of the receptor on tyrosine residues, thereby activating the intrinsic tyrosine kinase. Identification of a common signaling pathway used by both the insulin and IGF-I receptors has been established by the discovery of the insulin receptor substrate 1 (IRS-1) as the major intracellular substrate (Sun et al. 1991; Nishiyama and Wands 1992). Following tyrosyl phosphorylation of IRS-1 by the receptor tyrosine kinase, signaling molecules containing SH2 domains will bind to specific motifs located in the C-terminus of the molecule to form a multipotential signaling complex. These protein-protein interactions lead to amplification and diversification of the receptor-mediated signal. Previous studies have demonstrated that IRS-1 becomes highly tyrosyl phosphorylated during liver regeneration, leading to increased interactions with downstream signaling molecules (Sasaki et al. 1993). However, the role of other substrates of the insulin and IGF-I receptors such as IRS-2 , Gab-1 and $p62^{dok}$ (Sun et al. 1995; Holgado-Madruga et al. 1996; Yamanashi and Baltimore 1997), and Shc (Skolnik et al. 1993) in the promotion of liver growth and regeneration has not yet been established.

Ethanol has been shown to interfere with the IRS-1-mediated signal transduction pathway during liver regeneration induced by partial hepatectomy (Sasaki and Wands 1994). However, partial hepatectomy induces massive hepatocyte proliferation as a result of the simultaneous activation of numerous signal transduction pathways (Michalopoulos and DeFrances 1997). To study the specific effects of chronic ethanol exposure on the IRS-1-mediated signal transduction cascade in the setting of physiological concentrations of growth factors, we generated transgenic mice that overexpress the human IRS-1 gene exclusively in the liver (Tanaka et al. 1997). These transgenic mice exhibit increased levels of tyrosyl-phosphorylated IRS-1 protein, resulting in constitutive activation of the downstream mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) cascades and subsequent stimulation of hepatocyte proliferation. This transgenic mouse model was used to study the effects of dietary chronic ethanol consumption on the IRS-1-mediated signal transduction cascade in an attempt to define the mechanism(s) involved in the ethanol-induced inhibition of liver growth.

Insulin is an important modulator of growth and metabolic function in the central nervous system (CNS). Insulin is also a neurotropic factor, because it can support neuronal growth and differentiation in the absence of other growth factors (Haselbacher et al. 1985; Recio-Pinto et al. 1986; Baskin et al. 1987). In the CNS, insulin promotes neurite outgrowth, protein synthesis, neuronal cytoskeletal protein expression, and nascent synapse formation (Puro and Agardh 1984; Mill et al. 1985; Recio-Pinto et al. 1986; Heidenreich et al. 1988). The stimulatory effects of insulin are mediated through complex intracellular signaling pathways as described above. A virtually identical signaling cascade exists for IGF-I (Tartare et al. 1995). Insulin and IGF-I receptors are both abundantly expressed in the CNS (Havrankova et al. 1978; Goodyer et al. 1984; Gammeltoft et al. 1985; Haselbacher et al. 1985; Frank et al. 1986; Hill et al. 1986).

Ethanol has acute and chronic toxic effects on CNS function (Clarren et al. 1978; de la Monte 1988; Hunt 1993; Sanna and Harris 1993). Acute ethanol neurotoxicity is manifested by reversible alterations in neurotransmission and second messenger signaling (Saito et al. 1985; Gordon et al. 1986; Richelson et al. 1986; Diamond et al. 1987; Nagy et al. 1988; Tabakoff et al. 1988; Hoffman et al. 1989). Chronic ethanol neurotoxicity results in functional deficits associated with permanent structural injury (Jones et al. 1973; Hanson et al. 1976; Clarren et al. 1978; Streissguth et al. 1982; de la Monte 1988). In the mature brain, chronic ethanol exposure causes cerebral atrophy with reduced white matter volume, ventricular enlargement, and cerebellar atrophy with loss of Purkinje and granule cell neurons (Bauer and Altman 1975, 1977; Cragg and Phillips 1985; de la Monte 1988). Developmental exposure results in the fetal alcohol syndrome, which encompasses a broad array of neurological and systemic lesions, including CNS malformations such as microcephaly, reduced cerebral white matter volume, ventriculomegaly, cerebellar hypoplasia, and neuroglial heterotopias (Bauer and Altman 1975, 1977; Cragg and Phillips 1985). Thus, a major effect of chronic ethanol toxicity is loss of neurons and reduced white matter volume. The more extensive damage associated with fetal exposure suggests that the toxic effects of ethanol are greater in CNS cells that are metabolically active, proliferating, or differentiating, compared with CNS cells that are relatively quiescent.

Ethanol-induced CNS neurodegeneration and developmental arrest are probably mediated by the adverse effects of ethanol on intracellular signaling (Wands et al. 1979; Dow and Riopelle 1985; Saito et al. 1985; Gordon et al. 1986; Richelson et al. 1986; Diamond et al. 1987; Tabakoff et al. 1988; Resnicoff et al. 1994; Xu et al. 1995). Insulin and IGF-I signal transduction pathways that promote cell growth and viability in the CNS are important potential targets of ethanol toxicity because ethanol profoundly inhibits insulin and IGF-I signaling (Resnicoff et al. 1994; Xu et al. 1995), and insulin and IGF-I receptors are abundantly expressed in the CNS (Havrankova et al. 1978; Goodyer et al. 1984; Rosenfeld et al. 1984; Gammeltoft et al. 1985; Haselbacher et al. 1985; Frank et al. 1986; Hill et al. 1986). Previous studies demonstrated that ethanol inhibits growth factor receptor tyrosine kinase phosphorylation, activation of the intrinsic receptor tyrosine kinases, and stimulation of downstream signaling pathways (Duguay et al. 1982; Carter and Wands 1985; Wang et al. 1993; Bhavani et al. 1995; Xu et al. 1995). In addition, ethanol inhibits G protein expression (Diehl et al. 1992), cyclic adenosine monophosphate (cAMP)–dependent signaling, the association of PI3K with IRS-1 (Saito et al. 1985; Gordon et al. 1986; Richelson et al. 1986; Diamond et al. 1987; Nagy et al. 1988; Tabakoff et al. 1988), and second messenger cascades such as calcium phospholipid–dependent protein kinases (PKC) (Skwish and Shain 1990; Domenicotti et al. 1996; Steiner et al. 1997). PI3K is important for signaling cell survival through Akt/protein kinase B (Dudek et al. 1997; Eves et al. 1998). Therefore, ethanol inhibition of insulin signaling through PI3K may result in increased apoptosis of neuronal cells. The present study demonstrates that the growth-inhibitory effect of ethanol on insulin-stimulated PNET2 neuronal cells is due to increased apoptosis.

RESULTS

EFFECT OF ETHANOL ON THE LIVER

Expression and Tyrosyl Phosphorylation of IRS-1

The construction of transgenic mice overexpressing the human IRS-1 gene under the control of the albumin enhancer/promoter element has been previously described (Tanaka et al. 1997). Ethanol as well as isocaloric pair-fed control transgenic mice demonstrated high levels of human IRS-1 expression in the liver compared with nontransgenic littermates (figure 1A). More important, the extent of IRS-1 tyrosyl phosphorylation was found to be substantially reduced following chronic ethanol feeding for 8 weeks (figure 1B). Densitometric analysis of hepatic IRS-1 protein levels derived from three ethanol-fed and three pair-fed control transgenic mice revealed that expression was reduced by approximately 20 percent in ethanol-fed animals. However, tyrosyl phosphorylation of IRS-1 in liver lysates derived from chronic ethanol–fed transgenic mice was found to be substantially reduced (50 percent) compared with isocaloric pair-fed control mice. It is also noteworthy that the expression and tyrosyl phosphorylation of IRS-1 in the liver of nontransgenic littermates were very low, so it was not possible to accurately determine ethanol effects in the absence of the high levels of protein produced by expression of the human IRS-1 transgene. The biological consequences of reduced tyrosyl phosphorylation of IRS-1 in the liver of transgenic mice was further explored.

Effect of Ethanol on PI3K Activation

Tyrosyl-phosphorylated IRS-1 associates with the p85 kd subunit of PI3K, leading to activation of the 110 kd catalytic subunit. The association of IRS-1

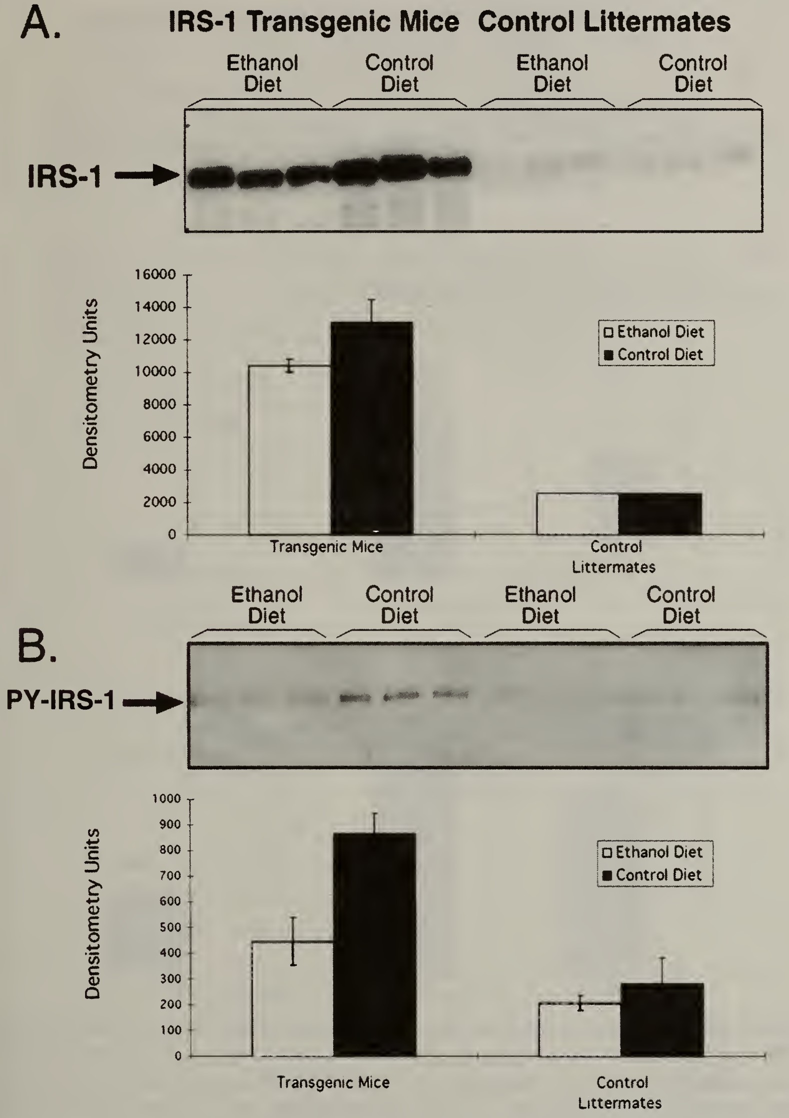

Figure 1. Expression and tyrosyl phosphorylation of insulin receptor substrate 1 (IRS-1) in the liver. (A) Levels of IRS-1 expression in the liver of IRS-1 transgenic mice fed ethanol and control diet compared with nontransgenic littermates. (B) Tyrosyl phosphorylation of IRS-1 (PY-IRS-1) was determined following immunoprecipitation with polyclonal anti-IRS-1 antibodies, followed by separation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS/PAGE) and immunoblotting with an anti-phosphotyrosine monoclonal antibody. Note the striking effect of chronic ethanol feeding on PY-IRS-1. Results of densitometric analysis are expressed as mean ± SEM.

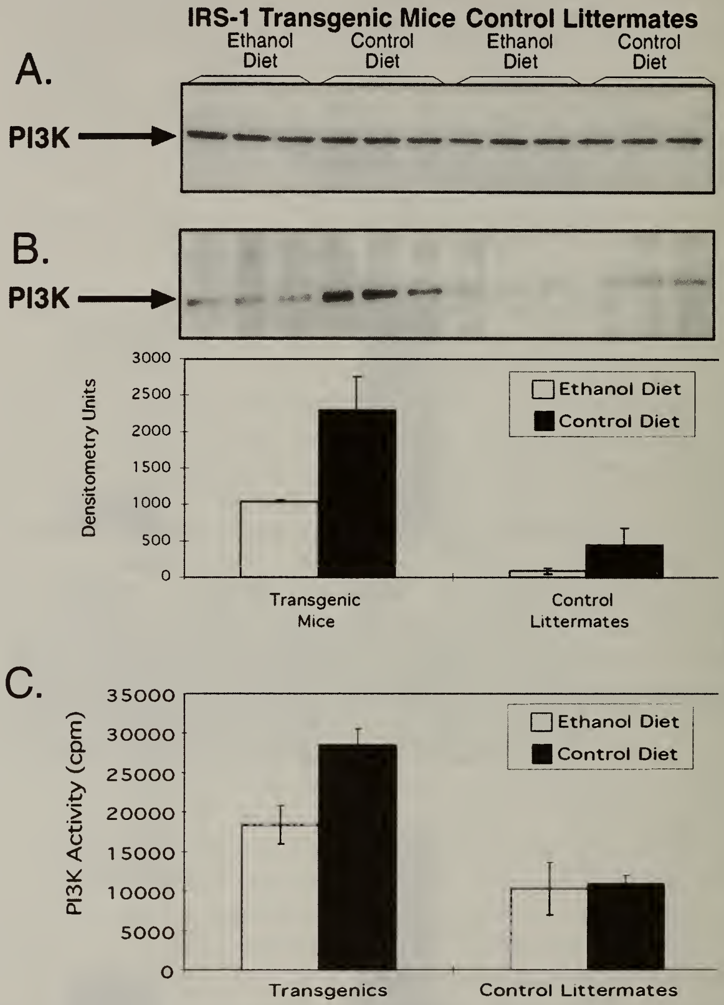

Figure 2. Effect of ethanol on phosphatidylinositol 3-kinase (PI3K) association and activation. (A) Western blot analysis demonstrates similar PI3K protein levels in liver lysates derived from animals receiving the ethanol and isocaloric control diets. (B) To analyze the association of PI3K with insulin receptor substrate 1 (IRS-1), liver lysates were immunoprecipitated with a polyclonal anti-IRS-1 antibody, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS/PAGE) and immunoblotted with a polyclonal antiserum against the p85 subunit of PI3K. There was reduced association of PI3K with IRS-1 in animals fed ethanol diet. (C) Activation of PI3K was measured following immunoprecipitation with polyclonal anti-IRS-1 antibody. Immunoprecipitates were reacted in a kinase buffer containing phosphatidylinositol and [^{32}P] γ-adenosine triphosphate. Extracted lipids were separated by thin layer chromatography and the amount of incorporated [^{32}P] was determined. There was a reduction in enzymatic activity of PI3K as well. Results are expressed as mean ± SEM.

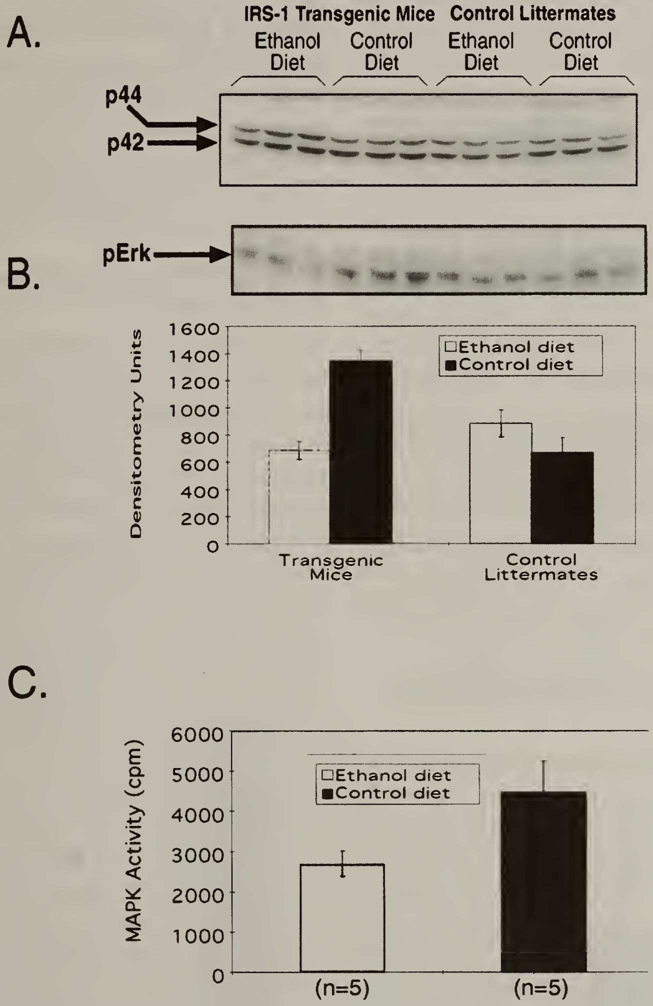

Figure 3. Phosphorylation and activation of mitogen-activated protein kinase (MAPK). (A) Western blotting with polyclonal anti-Erk antibodies demonstrated similar hepatic levels of Erkl (p44) and Erk2 (p42) in animals fed ethanol and isocaloric control diet. (B) For analysis of MAPK phosphorylation, the membrane shown in (A) was stripped and reprobed with a monoclonal antibody directed against activated MAPK, and the results were analyzed by densitometry. There was reduction of MAPK phosphorylation in ethanol-fed transgenic animals ($p < 0.01$). pErk = phospho-Erk. (C) Analysis of MAPK activation in insulin receptor substrate 1 (IRS-1) transgenic mice. Liver lysates were immunoprecipitated with a polyclonal antibody directed against Erk 2, washed extensively and followed by incubation in a kinase buffer containing $[^{32}P]$ γ-adenosine triphosphate and myelin basic protein. After separation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS/PAGE), the amount of $[^{32}P]$ incorporated into MBP was determined. Results are expressed as mean ± SEM.

with PI3K was evaluated in the liver of transgenic and nontransgenic littermates. Hepatic levels of PI3K were found to be similar in ethanol- and pair-fed control transgenic mice and nontransgenic littermates (figure 2A). We observed decreased binding of PI3K to IRS-1 in ethanol-fed transgenic animals (figure 2B). Similarly, activation of PI3K following binding to IRS-1, as determined by enzymatic assay, was significantly reduced in ethanol-fed compared with pair-fed control transgenic mice (figure 2C; 18,336 ± 2,414 vs. 28,443 ± 2,056 cpm, respectively, $p < 0.03$). In nontransgenic littermates, we were unable to demonstrate an ethanol effect on PI3K enzymatic activity following 8 weeks of chronic ethanol feeding (figure 2C, right side).

Phosphorylation and Activation of MAPK

Protein levels of p44 and p42 in liver lysates were not influenced by chronic ethanol feeding (figure 3A). However, phosphorylation of MAPK (pErk) as determined by immunoblot analysis was found to be significantly enhanced in pair-fed control transgenic animals compared with nontransgenic littermates, and this observation illustrates the biological effects of overexpression of the human IRS-1 transgene in the liver as shown in figure 3B ($p < 0.02$). This increased level of MAPK phosphorylation was found to be significantly reduced by chronic ethanol feeding and essentially returned to values observed in nontransgenic littermates ($p < 0.01$, figure 3B). To establish that the ethanol-induced reduction of MAPK phosphorylation also leads to impaired generation of MAPK activity, an enzymatic assay was performed as shown in figure 3C. It was found that the phosphorylation of myelin basic protein was reduced in chronic ethanol–fed compared with isocaloric pair-fed control transgenic mice.

Effect of Ethanol on Hepatocyte Proliferation

Measurement of [^{3}H]thymidine incorporation into liver DNA was used to assess the effect of chronic ethanol feeding on hepatocyte proliferation in both IRS-1 transgenic and nontransgenic littermates (figure 4). Hepatocyte DNA synthesis was substantially induced by expression of the IRS-1 transgene compared with nontransgenic littermates (621 ± 75 vs. 302 ± 88 cpm /500 µg DNA, respectively; $p < 0.001$). Chronic ethanol feeding resulted in a significant reduction of hepatocyte DNA synthesis compared with the isocaloric pair-fed control transgenic mice (244 ± 29 vs. 621 ± 75 cpm /500 µg DNA, respectively; $p < 0.003$). Indeed, hepatocyte proliferation in ethanol-fed transgenic animals was reduced to the basal level observed in pair-fed control nontransgenic littermates, indicating that ethanol feeding completely inhibited the enhanced DNA synthesis induced by the human IRS-1 transgene. In ethanol-fed nontransgenic animals, some reduction of [^{3}H]thymidine incorporation was also observed (statistically not significant), suggesting that chronic ethanol exposure reduces the physiological rate of hepatocyte proliferation as well. Thus, the striking inhibition of hepatocyte DNA synthesis observed in IRS-1 transgenic mice is due to specific effects of ethanol interfering with the IRS-1-mediated signal transduction pathway.

EFFECT OF ETHANOL ON NEURONAL CELLS

Effects of Ethanol on DNA Synthesis and Cell Growth

The control and ethanol-treated cultures had similar mean levels of [^{3}H]thymidine incorporation after 24 or 48 hours of insulin stimulation (figure 5). In contrast, cell density and net cell growth were significantly reduced in the ethanol-treated cultures as demonstrated by the crystal violet and MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide) assays. Control cultures exhibited stepwise growth, with near doubling of the mean cell density between the 24- and 48-hour time points. In the ethanol-treated cultures, the mean cell densities were 30 to 60 percent lower than in control cultures, and there was virtually no detectable net cell growth between the 24 and 48 hour time points, despite high levels of DNA synthesis (figure 5).

Ethanol-Induced Apoptosis

The reduced net cell growth vis-à-vis normal levels of DNA synthesis in ethanol-treated cultures suggested

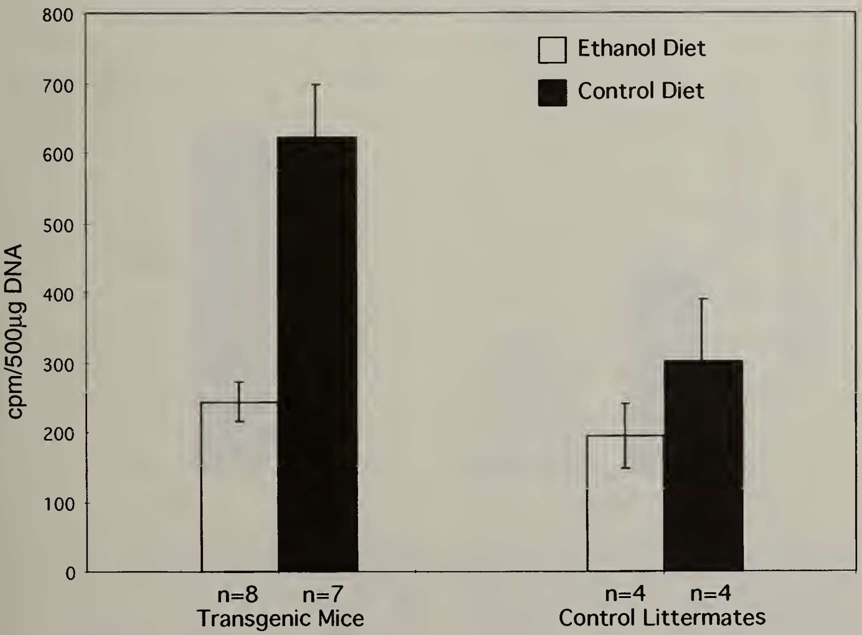

Figure 4. Effect of chronic ethanol feeding on hepatic DNA synthesis was determined by [^{3}H]thymidine uptake into 500 µg total liver DNA. DNA synthesis was significantly reduced in ethanol-fed insulin receptor substrate 1 (IRS-1) transgenic mice compared with control-fed animals ($p < 0.003$). Results are expressed as mean ± SEM.

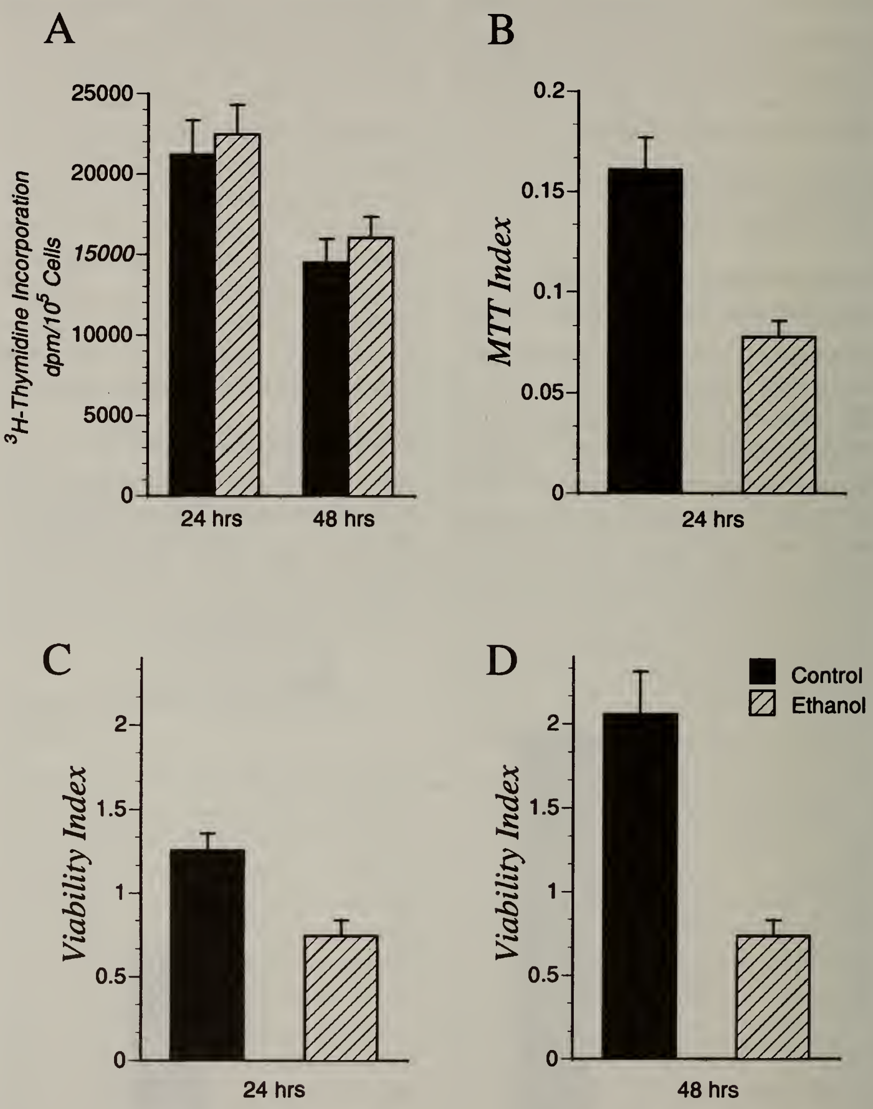

Figure 5. Effects of ethanol on DNA synthesis, viability, and growth in insulin-stimulated primitive neuroectodermal tumor type 2 (PNET2) neuronal cells. (A) [^{3}H] thymidine incorporation was measured 24 and 48 hours after insulin stimulation of serum-starved control and ethanol-treated cultures. dpm = disintegrations per minute. (B) Cell viability as measured by the MTT assay (see text) after 24 hours of insulin stimulation. (C and D) Cell viability as measured by crystal violet assays after 24 or 48 hours of insulin stimulation. The graphed data depict mean ± SD obtained for 12 replicate cultures. Each experiment was repeated at least three times with similar results.

that ethanol exposure diminished the viability of PNET2 cells. Therefore, several studies were conducted to determine whether ethanol exposure caused apoptosis of PNET2 cells. The cells were examined microscopically to detect changes in cytomorphology. The DNA content was measured in cultures harvested after overnight serum starvation and 24 or 48 hours of insulin stimulation. Agarose gel electrophoresis and Southern blot analysis of genomic DNA were used to detect DNA fragmentation ladders characteristic of apoptosis. Primer-independent [^{32}P]deoxycytidine triphosphate (dCTP) incorporation studies were used to quantify the relative abundance of nicked DNA. The TUNEL (terminal tranferase [14-biotin] dUTP end-labeling) assay was used to demonstrate nicked genomic DNA in situ.

The control cells exhibited uniform polygonal cytomorphology with abundant cytoplasm, and overnight serum starvation had no effect on cellular morphology. After 24 or 48 hours of insulin stimulation, greater than 95 percent of the control cells were adherent, flat, polygonal, and had short fine multipolar neurites (i.e., extended cell processes). Cells treated for 4–7 days with 100 mM ethanol in serum-containing medium were smaller than control cells and exhibited prominent radial extensions of irregular fine cell processes. Overnight serum starvation resulted in no detectable changes in cytomorphology. However, after 24 hours of insulin stimulation, neurite retraction and cell rounding occurred. After 48 hours of insulin stimulation, the ethanol-treated cultures contained numerous detached round, refractile cells, indicating reduced viability.

DNA quantification studies demonstrated stepwise increases in DNA content in control cultures and progressive reductions in DNA content in the ethanol-treated cultures (figure 6A). Electrophoresis of unlabeled genomic DNA revealed predominantly high-molecular-weight DNA in control cultures and conspicuous DNA fragmentation ladders in the ethanol-treated cultures (figure 6B). Southern blot analysis confirmed the presence of genomic DNA fragmentation in the ethanol-treated cells and exclusively high-molecular-weight DNA in control cells (figure 6B).

Corresponding with the results of Southern blot analysis, the [^{32}P]dCTP incorporation studies demonstrated increased labeling indices in ethanol-treated relative to control cultures (figure 6C). Insulin-stimulated control cells exhibited relatively low levels of [^{32}P]dCTP incorporation into genomic DNA. In the ethanol-treated cultures, the levels of [^{32}P]dCTP incorporation were significantly elevated by Student t test analysis ($p < 0.001$). In addition, the labeling indices were higher at the 48-hour time point, indicating progressive DNA fragmentation in the ethanol-treated cells. In control cultures, less than 1 percent of the cells exhibited nuclear condensation or fragmentation and associated TUNEL-positive nuclear staining. In contrast, in the ethanol-treated cultures, between 5 and 10 percent of the cells had condensed or fragmented TUNEL-positive nuclei after 24 hours

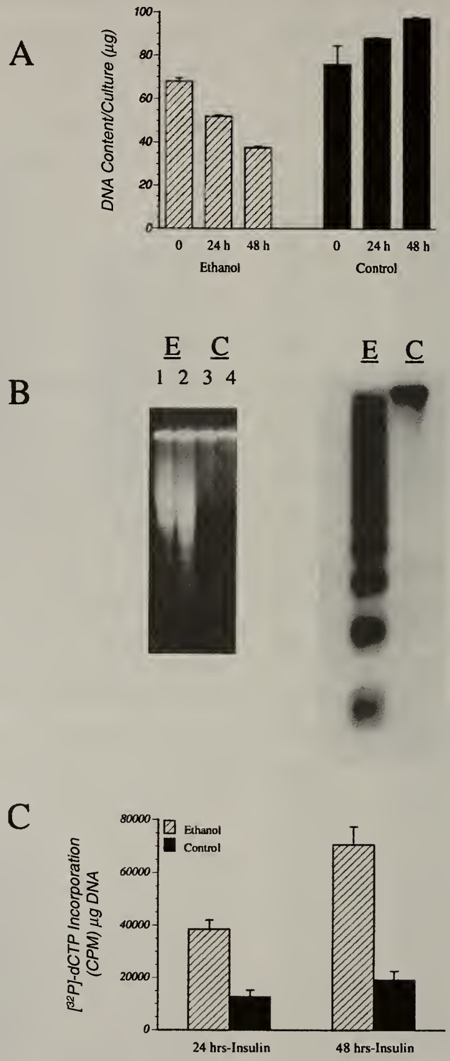

Figure 6. Progressive reductions in DNA content and increased nucleosomal DNA fragmentation following ethanol exposure and insulin stimulation. After 16 hours of serum starvation, the control and ethanol-treated cultures were stimulated with insulin (100 nM) for 0, 24, or 48 hours. (A) DNA was quantified using the 260-nm absorbance values. (B, left panel) Electrophoresis of ethanol-treated (E) and control (C) DNA samples (5 μg per lane) harvested after 24 (lanes 1 and 3) or 48 (lanes 2 and 4) hours of insulin stimulation. (B, right panel) Southern blot analysis of genomic DNA after 48 hours of insulin stimulation using probes generated with sonicated human genomic DNA. (C) Quantification of genomic DNA fragmentation and nicking by 3' end-labeling assays of [^{32}P]deoxycytidine triphosphate (dCTP) incorporation. CPM = counts per minute.

of insulin stimulation, and 10 to 20 percent were apoptotic after 48 hours of insulin stimulation (data not shown).

Increased p53 and Decreased bcl-2 Expression in Ethanol-Treated Cells

Previous studies of human neurodegenerative diseases demonstrated that apoptosis of CNS neurons is associated with increased p53 (de la Monte et al. 1997, 1998) and either decreased bcl-2 or increased Bax expression (Erro and Tunon 1997; Sawa et al. 1997; Su et al. 1997; Tortosa et al. 1998). In the present study, quantitative Western blot analysis was used to determine whether ethanol-induced apoptosis of PNET2 cells was mediated by altered expression of p53 and bcl-2. Both serum-starved and insulin-stimulated control cells had either undetectable or very low levels of p53 and high levels of bcl-2 expression, although the levels of bcl-2 were lower after serum starvation than after 18 or 24 hours of insulin stimulation. In addition, at the 48-hour time point, bcl-2 expression was again reduced.

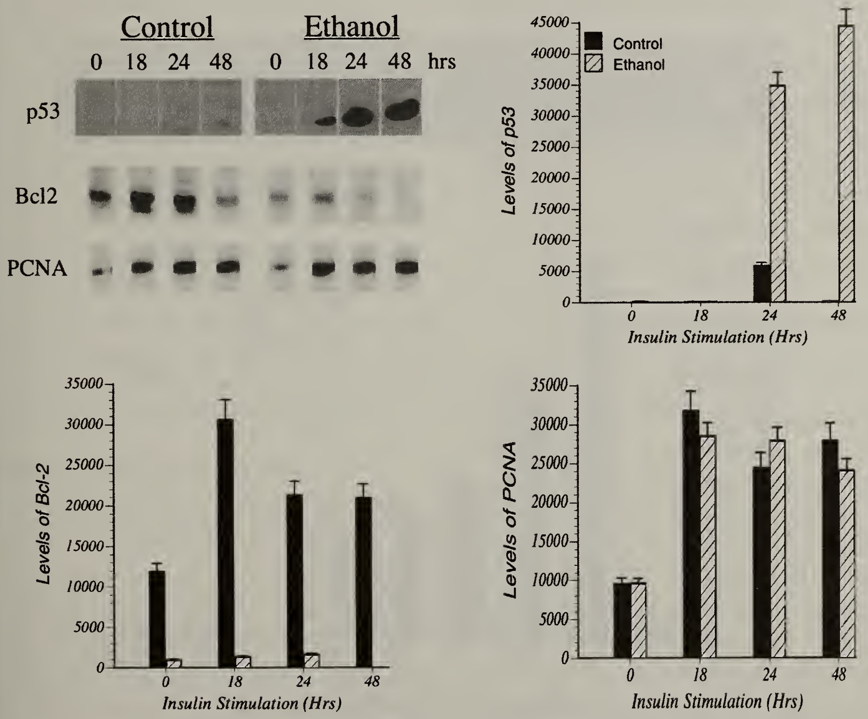

Figure 7. Analysis of p53 and Bcl-2 expression in control and ethanol-treated, insulin-stimulated (100 nM) cells. Cell lysates were analyzed by quantitative Western blot analysis. The panels at top left depict example results, and the graphs summarize data obtained from three replicate experiments. Values reflect arbitrary densitometry units (mean ± SD). PCNA = proliferating cell nuclear antigen.

Ethanol-treated, serum-starved cells had very low-level p53 expression, but with insulin stimulation, p53 expression increased strikingly and peaked at the 48-hour time point, coinciding with the period of maximum genomic DNA fragmentation (figure 7). In addition, the levels of bcl-2 in ethanol-treated cells were consistently lower than the levels in control cells, and at the 48-hour time point bcl-2 expression was absent. In contrast, control and ethanol-treated cells exhibited similar levels of proliferating cell nuclear antigen (PCNA) after serum starvation and insulin stimulation (figure 7).

Effects of Ethanol on Activated Erk MAPK

Insulin- or IGF-I-stimulated DNA synthesis is mediated by Erk MAPK activation (Porras et al. 1994; Welham et al.

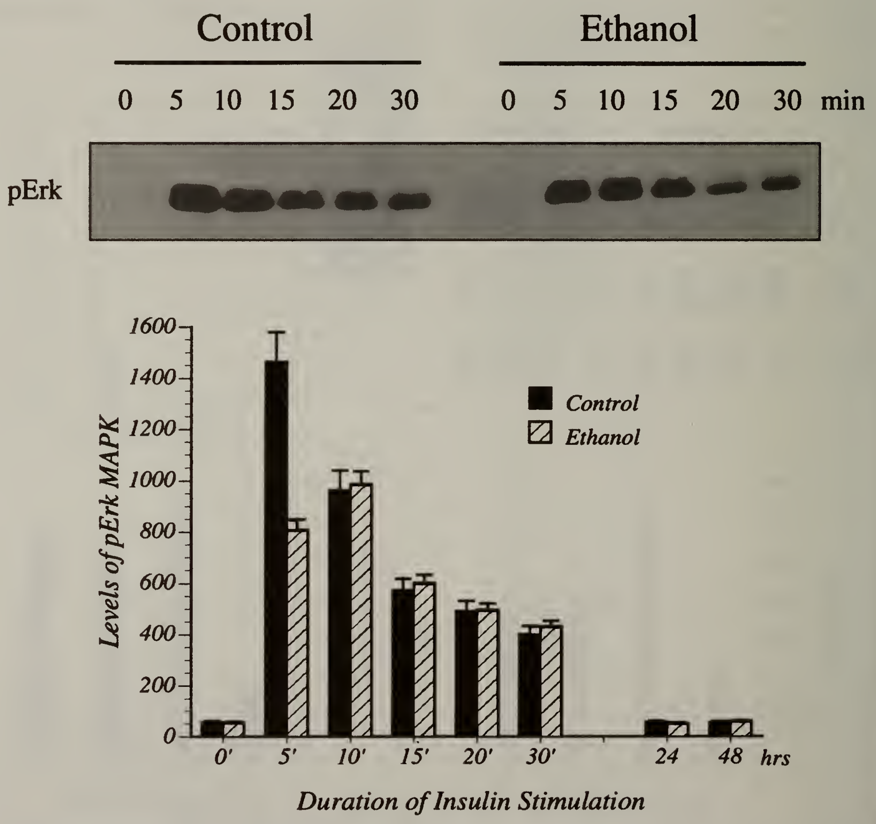

Figure 8. Activated Erk mitogen-activated protein kinase (MAPK) levels measured in insulin-stimulated control and ethanol-treated cells by Western blot analysis using phospho-specific antibodies. The levels of immunoreactivity were quantified by volume densitometry. The graph depicts results generated from three experiments, and the values reflect mean ± SD (arbitrary densitometry units). pErk = phospho-Erk.

1995; Porras and Santos 1996). In neuronal cells, Erk MAPK activity is also increased by growth factor–stimulated neuritic sprouting (Gomez and Cohen 1991; Garver et al. 1995). Quantitative Western blot analysis was used to assess the effects of ethanol on insulin-stimulated p42/p44 Erk MAPK activation using phospho-Erk–specific antibodies. Corresponding with the low levels of DNA synthesis and PCNA expression, active Erk MAPK was not detectable in serum-starved cultures, but within 5 minutes of insulin stimulation, the levels increased sharply in both control and ethanol-treated cells. Two notable differences in the ethanol-treated cells were (1) the peak levels of active Erk MAPK occurred after 10 rather than 5 minutes (control) of insulin stimulation, and (2) the peak levels of active Erk were reduced by 25 to 30 percent (figure 8). Otherwise, the profiles of Erk MAPK activation were similar for the two groups; in both groups the levels declined gradually between 10 and 30 minutes after adding insulin, and further marked reductions occurred after 24 and 48 hours of insulin stimulation.

Effects of Ethanol on Insulin-Stimulated IRS-1-Associated p85 kd Subunit of PI3K Activity

The interaction of tyrosyl phosphorylated IRS-1 (PY-IRS-1) with the p85 kd subunit of PI3K mediates metabolic signals that inhibit apoptosis (Rahn et al. 1994; Freund et al. 1995; Serve et al. 1995; Dudek et al. 1997; Suga et al. 1997; Summers et al. 1998). Various isoforms of the regulatory subunits of PI3K are expressed in the brain (Shin et al. 1998). Therefore, it was important to examine the levels of IRS-1-associated p85 kd subunit and PI3K activity following insulin stimulation (figures 9 and 10). Control and ethanol-treated cells exhibited very low levels of IRS-1-associated p85 kd subunit after overnight serum starvation (figures 9A and 9B). Within 5 minutes of insulin stimulation, control cells exhibited sharply increased and peak levels of IRS-1-associated p85. Between 10 and 30 minutes after insulin stimulation, the levels of IRS-1-associated p85 declined to the prestimulation levels. Ethanol treatment delayed and abbreviated the time course of insulin-stimulated increases in IRS-1-associated p85 (figures 9A and 9B). Ethanol treatment was associated with gradual rather than sharp increases in the levels of IRS-1-associated p85 and a delayed peak response (20 minutes). In contrast, the levels of total p85 were not modulated with insulin stimulation or ethanol treatment.

To determine if the reduced association of p85 with IRS-1 resulted in reduced PI3K activity, PI3K activity was measured in IRS-1 immunoprecipitants after 0, 5, or 20 minutes, and 24 or 48 hours of insulin stimulation (figures 10A and 10B). Serum-starved control and ethanol-treated cells had very low levels of IRS-1-associated PI3K activity. In control cells, PI3K activity increased abruptly, peaked after 5 minutes, and was still elevated after 20 minutes of insulin stimulation. After 24 or 48 hours of insulin stimulation, low but readily detectable PI3K activity was present in the control cells. In the ethanol-treated cultures, IRS-1-associated PI3K activity increased slightly after 5 minutes of insulin stimulation and peaked after 20 minutes of insulin stimulation (figure 10B), as confirmed by more detailed analysis of the time course (data not shown). In addition to the delayed peak response, ethanol treatment resulted in 60 to 70 percent reductions in the maximum level of insulin-stimulated PI3K activity, and 40 to 50 percent lower levels of PI3K activity after 24 or 48 hours of insulin stimulation (figure 10B).

DISCUSSION

ETHANOL EFFECTS ON LIVER GROWTH

The effect of acute and chronic ethanol exposure on liver regeneration has been studied using the well-characterized model of partial hepatectomy. Such investigations have demonstrated a potent inhibitory action of ethanol on liver regeneration as measured by hepa-

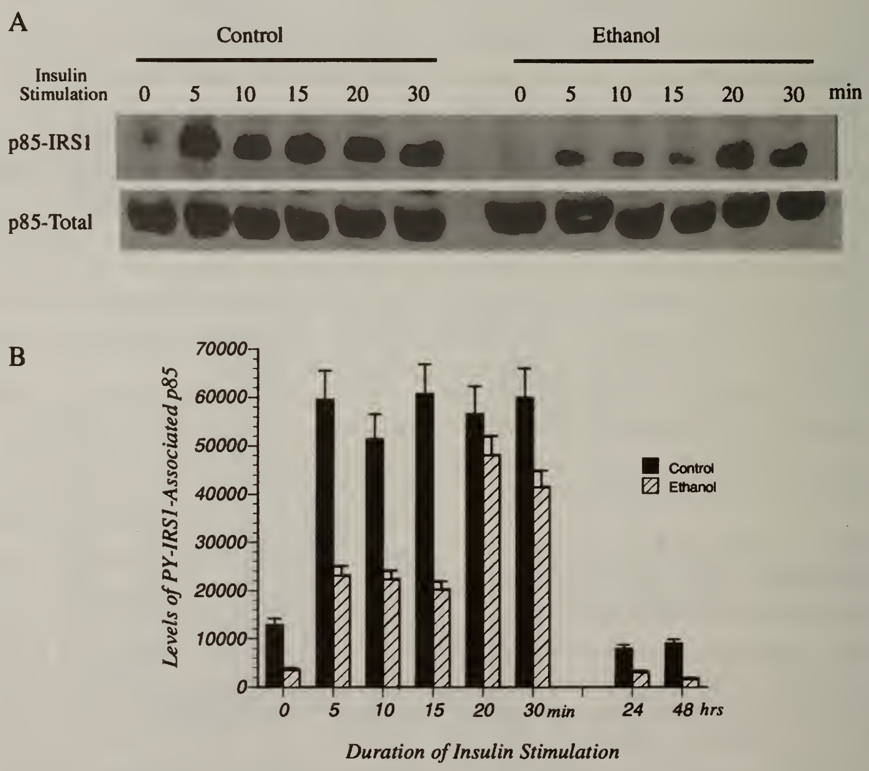

Figure 9. Analysis of insulin receptor substrate 1 (IRS-1)–associated p85 levels in insulin-stimulated control and ethanol-treated cells. IRS-1 immunoprecipitants were subjected to Western blot analysis using polyclonal antibodies to p85. Total p85 levels in the same lysates were simultaneously evaluated by direct Western blot analysis. The graphs depict the mean ± SD (densitometry units) of the IRS-1-associated p85 after insulin stimulation. The data were derived from three separate experiments. PY-IRS1 = tyrosyl phosphorylated IRS-1.

tocyte DNA synthesis (Wands et al. 1979; Diehl et al. 1988; Zhang et al. 1996). The role of a specific growth factor–induced signal transduction pathway in this regenerative process has been difficult to establish in this model system because of the simultaneous activation of several signaling cascades (Michalopoulos and DeFrances 1997).

In the present study, a transgenic mouse model was used for the first time to analyze the effect of chronic ethanol exposure on a specific signal transduction pathway in the liver. This model allowed us to investigate IRS-1-mediated signal transduction in the context of physiological concentrations of hepatic growth factors. Due to enhanced hepatocyte proliferation induced by the expression of the human IRS-1 transgene (Tanaka et al. 1997), it was possible to observe the

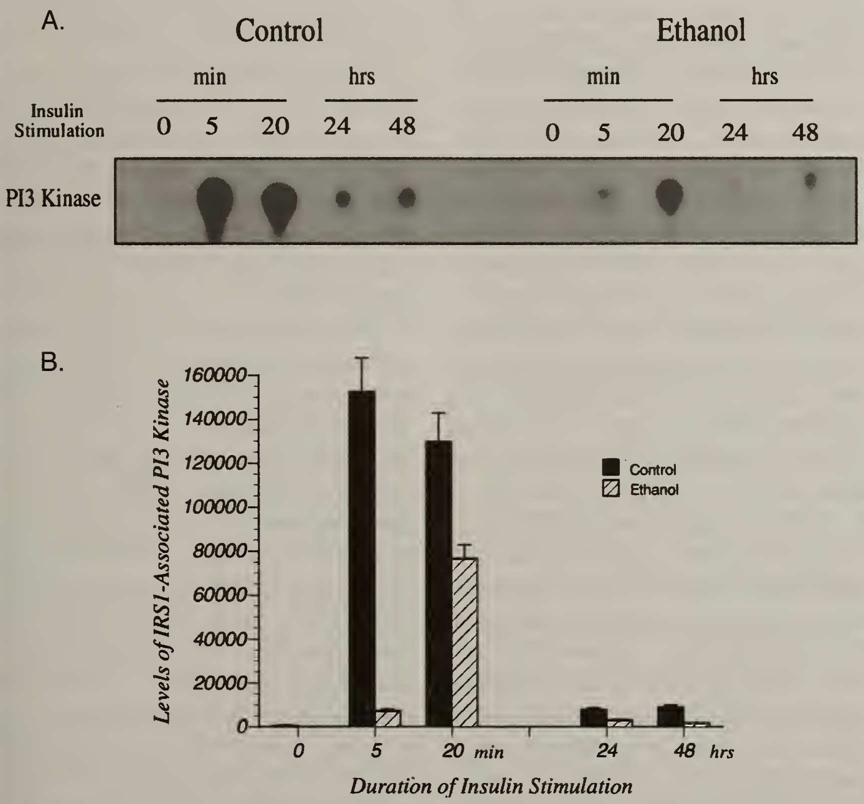

Figure 10. Analysis of insulin receptor substrate 1 (IRS-1)–associated phosphatidylinositol 3-kinase (PI3 kinase) levels in insulin-stimulated control and ethanol-treated cells. (A) PI3 kinase activity was measured in IRS-1 immunoprecipitants after 0, 5, or 20 minutes, and 24 or 48 hours of insulin stimulation. The autoradiogram represents [^{32}P]labeled phospholipids separated by thin-layered chromatography. (B) The graphs represent phosphoimager analysis of PI3 kinase levels (mean ± SD) from three replicate experiments.

influence of chronic ethanol feeding on selected downstream components of this growth factor–mediated signal transduction pathway and to establish this cascade as a major component in the regulation of hepatocyte proliferation. The findings in IRS-1 transgenic mice compared with nontransgenic littermates allowed us to determine specific ethanol effects on tyrosyl phosphorylation of proteins, subsequent protein-protein interactions, and downstream enzyme activation associated with cellular proliferation.

Indeed, IRS-1 is phosphorylated on tyrosine residues following cellular stimulation by insulin and IGF-I. Insulin had been shown to be essential for normal liver regeneration to occur (Bucher and Swaffield 1975; Starzl et al. 1976, 1978; Johnston et al. 1986; Chin et al. 1995; Knopp et al. 1997). Hepatic expression of the IGF-I receptor and the insulin-like growth factor binding protein-1 (IGFBP-1) is up-regulated following partial hepatectomy in rats (Caro et al. 1988; Mohn et al. 1991; Ghahary et al. 1992), indicating that IGF-I-mediated signaling may also promote liver regeneration. However, the observed increase of IRS-1 phosphorylation in the context of liver regeneration may not be entirely due to the effects of insulin and IGF-I, since several other growth factors and cytokines (e.g., interleukin-4, interleukin-9, interleukin-13, interferon-α, interferon-β, interferon-γ, growth hormone, leukemia inhibitory factor, and TNFα [Wang et al. 1993; Myers et al. 1994; Souza et al. 1994; Argetsinger et al. 1995; Welham et al. 1995; Yin et al. 1995; Guo and Donner 1996; Platanias et al. 1996]), have been shown to induce IRS-1 phosphorylation.

The tyrosyl-phosphorylated IRS-1 protein sends downstream signals by interaction with various SH2-containing molecules at specific motifs located in the C-terminal region of the protein. The Grb2 adapter protein recognizes the ^{895}EYVNIE motif of IRS-1, and the ^{1172}NYIDL motif binds to SHP2 tyrosine phosphatase (also known as Syp and SH-PTP2). The ^{608}GYMPMS, ^{939}EYMNMD, ^{460}NYICMG, and ^{987}DYMTMQ motifs are binding sites for the p85 kd subunit of PI3K. Binding of the p85 kd subunit to IRS-1 results in activation of the 110 kd catalytic subunit of PI3K. In addition, the small SH2/SH3 adapter proteins nck and crk also bind to tyrosyl-phosphorylated IRS-1 (White and Yenush 1998).

The N-terminal sequences contain a pleckstrin homology (PH) domain and a phosphotyrosine binding (PTB) domain (Gustafson et al. 1995), which couple the insulin receptor to IRS-1 (Myers et al. 1995; Yenush et al. 1996) Furthermore, the PH domain is involved in the IRS-1 interaction with the Janus tyrosine kinase Tyk-2 (Gustafson et al. 1995) Although functions of the PH domain remain to be clarified, demonstration of interactions with the G protein (β subunits) (Touhara et al. 1994) and phospholipids (Harlan et al. 1994) suggest that this region could play a role of linking IRS-1 to other signal transduction pathways.

Chronic ethanol feeding reduced the increased hepatocyte proliferation found in IRS-1 transgenic mice, thereby virtually abolishing the proliferative stimulus provided by overexpression of the human IRS-1 transgene (Tanaka et al. 1997). We further characterized the cellular events that may be responsible for this inhibitory effect of ethanol on IRS-1-induced liver growth. Tyrosyl phosphorylation of IRS-1 was found to be strikingly reduced. Inhibition of IRS-1 tyrosyl phosphorylation may represent a critical step in the alteration of downstream protein-protein interactions involved in this signal transduction pathway. One possible mechanism of ethanol action may be the down-regulation of receptor tyrosine kinase activity induced by several growth factors. Reduced tyrosyl phosphorylation of the insulin and IGF-I receptors has been previously reported for neuronal cell lines (Rosenfeld et al. 1984; Xu et al. 1995) and for NIH 3T3 cells (Resnicoff et al. 1993) following ethanol exposure in vitro. However, no difference was found with respect to binding of ligands to their receptors in the presence of ethanol (Fawcett et al. 1993; Resnicoff et al. 1993), suggesting that inhibition of downstream signal transduction events may be more important than ethanol effects exerted at the level of the receptor-ligand interaction. Indeed, a direct inhibitory effect of ethanol on autophosphorylation of purified IGF-I receptors was demonstrated following IGF-I stimulation (Resnicoff et al. 1993). Ethanol exposure may also influence IGF-I synthesis (Srivastava et al. 1995) Although we were unable to demonstrate a significant effect of chronic ethanol feeding on tyrosyl phosphorylation of the β subunit of the insulin receptor, other extracellular signaling molecules are known to contribute to tyrosyl phosphorylation of IRS-1 (Wang et al. 1993; Souza et al. 1994; Argetsinger et al. 1995; Yin et al. 1995; Guo and Donner 1996). In this regard, TNFα and growth hormone–induced signals have been found to be inhibited by ethanol exposure (Akerman et al. 1993; Xu et al. 1995; Zeldin et al. 1996).

Tyrosyl phosphorylation of IRS-1 results in the activation of two major downstream signal transduction pathways, namely the MAPK and PI3K cascades. Tyrosyl-phosphorylated IRS-1 interacts with the SH2 domains of Grb2 and Syp (SH-PTP2) proteins, and these events result in the activation of Ras (Skolnik et al. 1993) Ras-GTP (guanosine triphosphate) subsequently interacts with Raf-1, recruiting it to the cell membrane where Raf-1 becomes activated as well (Marshall 1995) and leads to the sequential downstream activation of mitogen-activated protein kinase kinase (MAPKK) and the MAPK cascade. Indeed, activated MAPKs are associated with cellular proliferation and differentiation. In our experiments, simple overexpression of a normal signal transduction molecule high up in the signal transduction cascade was sufficient to increase MAPK activity in IRS-1 transgenic mice compared with nontransgenic littermates. Furthermore, this enhanced MAPK activity was reduced to basal levels found in the liver of nontransgenic littermates when mice were placed on a chronic ethanol diet. Since constitutive MAPK activation in the liver is associated with hepatocyte proliferation (Tanaka et al. 1997), this action of ethanol may represent a possible molecular mechanism by which ethanol impairs liver growth.

Binding of the regulatory p85 kd subunit of PI3K to tyrosyl-phosphorylated IRS-1 results in activation of the 110 kd catalytic subunit (Myers et al. 1994). The activation of PI3K by insulin and IGF-I may account for many of the known metabolic responses of cells to these growth factors (Okada et al. 1994). Recent studies have demonstrated the importance of PI3K signaling in the prevention of apoptosis following insulin and IGF-I stimulation in fibroblasts (Kauffmann et al. 1997) and neuronal cell lines (Yao and Cooper 1995; Dudek et al. 1997). Activation of protein kinase B (PKB) (Akt) by PI3K appears to be the major downstream mediator of cell survival. Hu and colleagues (1995) demonstrated that Ras-dependent cellular responses were enhanced by constitutively activated PI3K. In this respect, binding of the p85 kd subunit of PI3K to IRS-1 induced PI3K activity in transgenic mice, and both binding and enzymatic activation of PI3K were found to be significantly impaired by chronic ethanol feeding. This observed inhibition of PI3K activity by ethanol may lower the threshold for apoptotic cell death as well as reduce hepatocyte proliferation, which further illustrates the potential pleiotropic actions of ethanol on the liver.

We also observed a slight reduction of IRS-1 protein levels in the liver of chronic ethanol–fed transgenic animals. The reduction of IRS-1 protein concentrations occurred despite similar levels of expression of other cellular proteins involved in the signal transduction cascade, such as PI3K, Erk1, and Erk2. The transgene was placed under the transcriptional control of the human albumin promoter, which has not been found to be negatively regulated by chronic ethanol exposure in previous studies (Zern et al. 1983; Annoni et al. 1990). Posttranscriptional regulation may have influenced the half-life of the IRS-1 transcripts in the context of chronic ethanol feeding. Nevertheless, direct comparison of the extent of protein expression to the levels of tyrosyl-phosphorylated IRS-1 revealed that reduced IRS-1 phosphorylation in ethanol-fed animals was far in excess of that due to slightly lowered protein levels.

Inhibition of the effects of growth factors that use the IRS-1 signal transduction cascade may substantially influence the regenerative capacity of hepatocytes. It will be of interest to determine if ethanol acts directly or whether toxic metabolites such as acetaldehyde or free radical formation generated during chronic ethanol exposure (Shaw et al. 1995) inhibit this signal transduction cascade involved in hepatocyte growth. Furthermore, the role of other IRS proteins (Sun et al. 1995; Holgado-Madruga et al. 1996; Yamanashi and Baltimore 1997) in the context of ethanol-induced inhibition of liver growth needs to be defined. Finally, this study emphasizes that transgenic mouse models are powerful tools to examine the inhibitory effect of chronic ethanol consumption on hepatocyte proliferation and to further define molecular mechanisms involved in this process.

Ethanol has potent inhibitory effects on insulin signal transduction pathways in many cell types, including neurons and glial cells (Wands et al. 1979; Duguay et al. 1982; Carter and Wands 1985; Resnicoff et al. 1994; Bhavani et al. 1995; Xu et al. 1995). For example, ethanol impairs insulin and IGF-I signaling through IRS-1 by inhibiting tyrosyl phosphorylation of the receptors, activation of the receptor tyrosine kinases, and tyrosyl phosphorylation of IRS-1 (Bhavani et al. 1995; Xu et al. 1995), with attendant reduction in MAPK activity (Banerjee et al. 1998; Mohr et al. 1998). Ethanol inhibition of insulin and IGF-I signaling also results in reduced levels of IRS-1-associated p85 and PI3K activity (Sasaki et al. 1993; Resnicoff et al. 1994; Sasaki and Wands 1994; Zhang et al. 1998). PI3K is an important mediator of neuronal survival in the CNS (Shin et al. 1998). Therefore, the inhibitory effects of ethanol on insulin and IGF-I signaling can lead to impaired DNA synthesis, metabolic activity, or cell survival mechanisms (Carter and Wands 1985, 1988; Resnicoff et al. 1994; Bhavani et al. 1995; Xu et al. 1995).

Effect of Ethanol on Signal Transduction Pathways in Neuronal Cells

Ethanol inhibits proliferation of some neuronal or glial cells by delaying mitosis, prolonging the mitotic cycle, or reducing the number of neurons initiating DNA synthesis (Miller 1989; Resnicoff et al. 1994). Ethanol inhibition of DNA synthesis and cell proliferation (Carter and Wands 1985, 1988; Diehl et al. 1992) is due to impaired insulin or IGF-I signaling through IRS-1 (Sasaki et al. 1993; Sasaki and Wands 1994; Bhavani et al. 1995) and Erk MAPK (Banerjee et al. 1998; Mohr et al. 1998). In the present study, the ethanol-exposed PNET2 cells exhibited reduced peak levels of phospho-Erk after 5 minutes of insulin stimulation. However, this effect did not result in decreased DNA synthesis or PCNA expression. A similar result was obtained recently with respect to IGF-I-stimulated cerebellar granule cells (Zhang et al. 1998). Therefore, the adverse effects of ethanol on growth factor signaling does not always lead to impaired DNA synthesis in neuronal cells. Instead, ethanol inhibition of cell growth may be due to reduced viability and increased apoptosis as demonstrated herein.

Increased apoptosis in ethanol-exposed, insulin-stimulated PNET2 neuronal cells was manifested by reduced cell viability, increased nucleosomal DNA fragmentation on agarose gels (laddering), and increased [^{32}P]dCTP or [11-biotin]dCTP incorporation into nicked genomic DNA. Ethanol-induced neuronal apoptosis was preceded by increased p53 and decreased bcl-2 expression and reduced levels of PI3K activities. In a recent study, ethanol-induced apoptosis of IGF-I-stimulated neuronal cells was also found to be mediated by inhibition of PI3K activity (Zhang et al. 1998). The present study demonstrates that the activation of apoptosis and apoptosis signaling pathways in ethanol-treated cells requires growth factor stimulation.

Ethanol-induced apoptosis of PNET2 neuronal cells was associated with increased p53 and decreased bcl-2 expression. Of note is that the reduced cell viability observed throughout the period of study was associated with decreased levels of bcl-2 and minimally increased p53, whereas the large-scale apoptosis observed after 24 or 48 hours of insulin stimulation was associated with abrupt and strikingly increased levels of p53. This suggests that the steady cell loss associated with chronic ethanol exposure may have been due to reduced viability, whereas the massive apoptosis observed after 24 or 48 hours of insulin stimulation was probably precipitated by cellular accumulation of p53. It has been shown that p53 has a role in neuronal and glial cell apoptosis (Eizenberg et al. 1995, 1996; de la Monte et al. 1997, 1998), and high-level bcl-2 expression mediates neuronal survival (Behl et al. 1993; Zhong et al. 1993; Motoyama et al. 1995).

Increased levels of p53 occurred only in the ethanol-treated cells that had been stimulated with insulin. Unlike growth factor withdrawal, insulin stimulation was required to activate pro-apoptosis genes and intracellular signaling. The mechanism by which insulin stimulation of ethanol-exposed cells results in p53 induction is not known. Ethanol inhibits insulin-stimulated DNA synthesis by reducing tyrosyl phosphorylation of the IRS-1 and attendant binding to SH2-containing molecules such as Grb2 and Syp. Although this pathway is adversely affected by ethanol in many cell types, our studies indicate that it is not impaired in ethanol-exposed PNET2 cells, since the levels of active Erk MAPK and DNA synthesis were similar to control. This suggests that IRS-1 binding to SH2-containing molecules may not be substantially reduced in insulin-stimulated ethanol-treated neuronal cells. In contrast, the marked inhibitory effect of ethanol on IRS-1-associated p85 and PI3K activity indicates that insulin-stimulated tyrosyl phosphorylation of the YMXM motif of IRS-1 is substantially inhibited in ethanol-exposed neuronal cells. Importantly, the inhibitory effect of ethanol on insulin signaling in PNET2 neuronal cells appears to be selective rather than global, as demonstrated recently in FOCUS hepatocellular carcinoma cells (Banerjee et al. 1998).

Ethanol-induced neuronal apoptosis was associated with reduced association of p85 with PY-IRS-1 and reduced levels of PI3K activity. Therefore, inhibition of growth factor–stimulated PI3K activity is probably the major factor mediating apoptosis in ethanol-exposed neuronal cells. Ethanol-induced apoptosis of IGF-I-stimulated cerebellar granule neurons is also linked to inhibition of PI3K activity (Zhang et al. 1998). Downstream effectors of PI3K include Rac1, PKC, and the serine-threonine kinase Akt (PKB). Akt is the effector molecule that mediates survival of a number of cell types (Kauffmann et al. 1997; Khwaja et al. 1997; Kulik et al. 1997), including neurons (Eves et al. 1998). Both insulin and IGF-I activate Akt (Kulik et al. 1997) through PI3K signaling (D'Mello et al. 1997). Chemical inhibition of PI3K activity by wortmannin or LY294002 induces neuronal apoptosis (D'Mello et al. 1997) due to inhibition of Akt/PKB. Together, these findings suggest that inhibition of insulin and IGF-I signaling through PI3K is an important mechanism of ethanol-induced apoptosis of neuronal cells.

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Chapter 7

Ethanol-Induced Inhibition of Insulin-Like Growth Factor I Signaling

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KEY WORDS: ethanol; biological inhibition; insulin; growth promoting factors; cell signaling; chronic AODE (effects of AOD [alcohol or other drug] use, abuse, and dependence); tissue; cell growth and differentiation; biological regulation; gene expression

EFFECT OF ALCOHOL ON CELL PROLIFERATION AND APOPTOSIS

Alcoholism is associated with profound degenerative conditions affecting many organ systems, including myopathy, neuropathy, anemia, bone loss, and cirrhosis. Newborns afflicted with fetal alcohol syndrome display extensive loss of neurons, resulting in a conspicuous decrease in the size of many regions of the brain, and have distinctive craniofacial and cardiac abnormalities (Miller and Potempa 1990; Schenker et al. 1990; Miller 1992).

Using Occams's razor, one is drawn toward a unitary hypothesis to explain ethanol toxicity. However, given the numerous potential cellular targets of ethanol, a single critical site responsible for ethanol-induced tissue damage is unlikely. Indeed, several features of alcoholic disease suggest that there may be overlapping sets of primary and secondary metabolic events modified by host factors that lead to specific tissue damage. First, ethanol is an exceedingly weak toxin both in vivo and in vitro. Second, the patterns of organ damage are not consistent among individual alcoholics. Third, tissue damage occurs only after a lag phase, and is often episodic. Taken together, these observations raise the possibility that exogenous or endogenous cofactors are required to elicit tissue damage in the setting of alcoholism. Stated differently, ethanol

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may sensitize target organs toward otherwise indolent stresses.

Some of the long-term deleterious effects of ethanol may relate to its effect on cell proliferation. In most studies, ethanol is an inhibitor of cell growth. Ethanol inhibits liver cell regeneration, which may play a role in the development of cirrhosis (Wands et al. 1979; Duguay et al. 1982). In fetal alcohol syndrome, the loss of neuroglial cells may relate to inhibited cell proliferation (Miller 1992). The growth of neuronal cells in vitro is sensitive to inhibition by ethanol (Isenberg et al. 1992; Resnicoff et al. 1994; Luo and Miller 1998). Other cells that are growth-inhibited by ethanol, at least in vitro, include fibroblasts (Resnicoff et al. 1993), osteoblasts (Friday and Howard 1991), hematopoietic cells (Imperia et al. 1984; Cook et al. 1990), and glial cells (Davies and Cox 1991; Snyder et al. 1992; Kane et al. 1996). Recent studies have demonstrated that the interaction of ethanol with specific growth factor signal transduction pathways may account for suppressed growth. In this chapter we present the evidence that the insulin-like growth factor I (IGF-I) signaling pathway is particularly sensitive to ethanol.

APOPTOSIS AND ALCOHOL-INDUCED TISSUE DAMAGE

It is likely that tissue damage in alcoholism occurs by apoptosis. This is manifest, for example, in the heart, muscles, and nervous system by single cell dropout. Similarly, neuronal apoptosis in fetal alcohol syndrome has been suggested in several experimental settings (Kentroti and Vernadakis 1991; Cartwright and Smith 1995). There is a close relationship between cell cycle progression and apoptosis. Interruption of the cell cycle at critical checkpoints generally promotes apoptosis induced by exogenous influences, perhaps as a default pathway (Meikrantz and Schlegel 1995). A similar phenomenon occurs when cells prematurely enter the cell cycle (Evan et al. 1992). In this context, several growth factors and cytokines not only promote cell cycle progression but also provide trophic support as inhibitors of apoptosis.

This background offers a potential model of ethanol-induced tissue injury wherein the antiproliferative effects of ethanol may enhance cellular sensitivity to apoptosis. That is, the inhibition of growth factor signaling by ethanol may sensitize cells to apoptosis by interfering with cell cycle progression, or by directly reducing those intracellular signals that mediate cell survival. This conjecture introduces a role for cofactors in the induction of apoptosis in the presence of ethanol, as well as the episodic nature of tissue damage.

REGULATION OF CELL GROWTH AND APOPTOSIS BY IGF-I

The IGF-I receptor (IGF-IR) is a critical determinant of cell cycle progression in many cell types (LeRoith et al. 1995 and references therein; Rubin and Baserga 1995). IGF-I, IGF-II, and insulin activate the IGF-IR, although under physiological conditions, IGF-I binds with the highest efficiency and likely exerts most of the biological actions of the IGF-IR. The biological actions of the IGF-I/IGF-IR signaling axis have been reviewed extensively (Lowe 1991). Briefly, many cell types require IGF-I for survival and growth in vitro, including neuroglial cells, hematopoietic cells, smooth-muscle cells, osteoblasts, and keratinocytes. IGF-I and IGF-II exert growth-promoting effects in various regions of the brain, including the telencephalon, hippocampus, cerebellum, and spinal motoneurons. IGF-I levels correlate closely with body size. Targeted disruption of the IGF-I in mice results in profound reduction in birth weights and adult size. Conversely, IGF-I transgenic mice exhibit enhanced growth. IGF-I also contributes to tissue repair and regeneration, a fact that has led to the investigation of therapeutic uses of IGF-I.

IGF-I is a critical factor in the progression of cells from G_1 to S phase. For example, in fibroblasts, platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) are required for induction of competence, but S phase progression requires IGF-I. This feature of S phase transition is amply evidenced by the inability of mouse fibroblasts with targeted disruption of the IGF-IR (R^- cells) to enter S phase in defined medium supplemented with an array of growth factors. Autocrine growth regulation of many tumor cell types is mediated at least in part by the disruption of IGF-IR binding or by reduction of the IGF-IR expression by ribonucleic acid (RNA) antisense technology (Resnicoff et al. 1995).

In addition to the growth-promoting activities of the IGF-IR, the IGF-IR is a critical determinant of the survival of cells. IGF-I directly prevents apoptosis following cell injury. Importantly, R^- cells, or wild-type cells that have been treated with IGF-IR antisense oligodeoxynucleotides, rapidly undergo apoptosis when exposed to the in vivo environment (Resnicoff et al. 1995). IGF-I promotes in vitro and in vivo survival of primary cultured and cloned neuroblastoma cell lines (Bozyczko-Coyne et al. 1993; LeRoith et al. 1993; Beck 1994). In neuronal cells, apoptosis induced by various injuries such as ischemia and hyperosmosis is directly prevented by IGF-I (Gluckman et al. 1992; Matthews et al. 1997).

IGF-I RECEPTOR

The IGF-IR is homologous to the insulin receptor and exists as an $\alpha2$-$\beta2$ heterodimer, the individual chains linked by α-α and α-β disulfide bridges (Rubin and Baserga 1995) (figure 1). The extracellular ligand binding domain is constituted on the α chain, and the tyrosine kinase domain is on the intracellular β domain. The tyrosine kinase domain is over 80 percent homologous to the insulin receptor tyrosine kinase and is poorly homologous within the carboxy terminal domain. Upon ligand binding, the receptor undergoes autophosphorylation within the tyrosine kinase domain (tyrosines 1131, 1135, and 1136). Other key tyrosines are located at Y950, Y1250, Y1251, and Y1316. The role of the respective tyrosines in cell proliferation and survival has been dissected by means of site-directed mutagenesis, and has been reviewed elsewhere (Baserga et al. 1997).

The activated IGF-IR binds and phosphorylates several signaling molecules, notably IRS-1 (insulin receptor substrate 1) and Shc (see figure 1). IRS-1 possesses numerous SH domains that dock SH group–containing adaptor molecules. Importantly, IRS-1 and Shc bind Grb2, which leads to the activation of Ras and consequently mitogen-activated protein (MAP) kinase. IRS-1 also binds phosphatidylinositol 3-kinase (PI3K). It has been demonstrated that PI3K activation is a major pathway for the inhibition of apoptosis via the activation of protein kinase B/Akt, and subsequent phosphorylation of BAD (Datta et al. 1997; del Peso et al. 1997; Dudek et al. 1997). Other substrates for IGF-IR tyrosine kinase include Crk and Vav, and focal adhesion kinase.

EFFECT OF ETHANOL ON IGF-I SIGNALING

The mouse fibroblast cell line BALB/c3T3 has been used as model for the

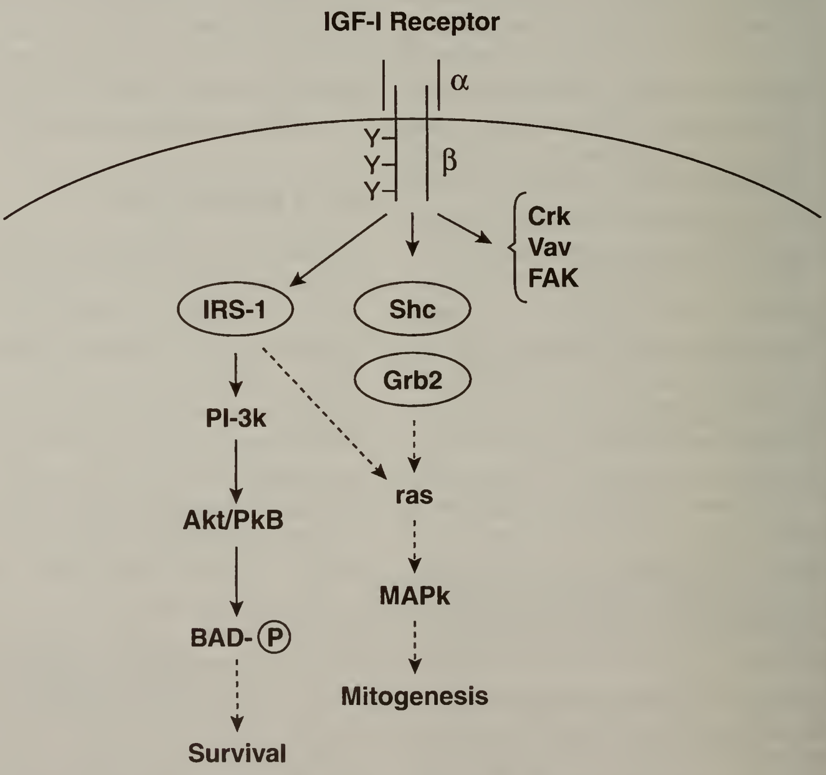

Figure 1. Pathways of insulin-like growth factor I (IGF-I) receptor-mediated mitogenesis and survival. FAK = focal adhesion kinase; IRS-1 = insulin receptor substrate 1; PI-3k = phosphatidylinositol 3-kinase; PkB = protein kinase B; MAPk = MAP kinase; BAD-P = phosphorylated BAD.

effect of ethanol on cell growth (Resnicoff et al. 1994, 1996). In serum-free medium, competence is achieved by the addition of PDGF, but S phase progression and mitogenesis require IGF-I. Ethanol inhibits IGF-I-dependent cell growth in both wild-type BALB/c3T3 cells and in cells that overexpress the human IGF-I receptor (p6 cells). The growth-suppressive effect of ethanol is accompanied by an inhibition of IGF-I-induced proto-oncogene induction (c-*fos*, c-*jun*, and c-*myc*). Half-maximal effective concentration of ethanol for growth suppression is about 50 mM. The inhibitory effect is specific for IGF-I. Ethanol had no effect on the induction of competence by PDGF or EGF. However, mitogenesis was inhibited by ethanol when it was added exclusively during the period of IGF-I stimulation.

The inhibition of IGF-I-dependent cell proliferation by ethanol has been demonstrated in several neuroglial cell types, including C6 rat glioblastoma cells (Resnicoff et al. 1994), astrocytes (Snyder et al. 1992; Kane et al. 1996), and several neuroblastoma cell lines (Luo and Miller 1997*a*, 1997*b*). Wands and colleagues (Xu et al. 1995) have demonstrated inhibitory effects of ethanol on insulin signaling in primitive neuroectodermal tumor (PNET) cells. The role of the IGF-IR in this process was not investigated.

In studies of C6 rat glioblastoma cells, ethanol also inhibited EGF-dependent cell proliferation (Resnicoff et al. 1994). However, at least one mechanism by which EGF promotes mitogenesis is by induction of IGF-IR expression leading to IGF-I autocrine stimulation. We have demonstrated this to be the case for C6 cells, which secrete IGF-I. Autocrine growth can be inhibited by IGF-IR antisense oligodeoxynucleotides (Resnicoff et al. 1994). In C6 cells, EGF stimulated an increase in IGF-IR expression. The mitogenic response to EGF was also inhibited by the IGF-IR antisense techniques. Ethanol had no effect on IGF-IR induction in response to EGF. Thus, we have proposed that ethanol interferes with EGF signaling in C6 cells by interfering with subsequent IGF-I autocrine growth.

ETHANOL INHIBITS IGF-IR TYROSINE AUTOPHOSPHORYLATION

In p6 cells, ethanol inhibits the IGF-I-dependent tyrosine autophosphorylation of the IGF-IR (Resnicoff et al. 1993). The reduced tyrosine phosphorylation of the IGF-IR corresponds to variable inhibition of intracellular IGF-I signaling including the tyrosine phosphorylation of IRS-1 phosphorylation (Resnicoff et al. 1994), p42/p44 MAP kinase (Seiler et al. unpublished data), and proto-oncogene induction (c-*jun*, c-*fos*, c-*myc*) (Resnicoff et al. 1993). Similar inhibitory effects of ethanol on IRS-1 phosphorylation have been observed in hepatocytes (Mohr et al. 1998) and rat cerebellar granule cells (Zhang et al. 1998*a*, 1998*b*).

Ethanol may directly inhibit the tyrosine autophosphorylation of the IGF-IR. Studies have been performed largely with the human IGF-IR isolated from p6 cells. IGF-IR tyrosine autophosphorylation was inhibited by ethanol in response to both IGF-I and IGF-II. Importantly, ethanol inhibits the autophosphorylation of the immunopurified IGF-I receptor preparations (Resnicoff et al. 1993). Ethanol also inhibits tyrosine kinase activity toward exogenous substrate using receptors prepared by wheat germ lectin chromatography (Rubin et al. unpublished data).

Wands and colleagues have identified IRS-1 as a target for ethanol inhibition (Xu et al. 1995; Mohr et al. 1998). In PNET cells (Xu et al. 1995) and hepatocytes (Banerjee et al. 1998), ethanol also decreased insulin receptor tyrosine autophosphorylation, but IGF-IR autophosphorylation was not measured. One possibility is that ethanol may disrupt the association of both the insulin receptor and IGF-IR with IRS-1, leading in some manner to a reversal of receptor autophosphorylation. Alternatively, both the insulin receptor and the IGF-IR tyrosine kinases may be targets for ethanol in these cells.

EFFECT OF ETHANOL ON APOPTOSIS

The recognition that ethanol inhibits IGF-IR activation led to the hypothesis that ethanol might enhance apoptosis by repressing the survival function of IGF-I. This was originally tested in mouse fibroblast culture (Cui et al. 1997). 3T3 cells are resistant to tumor necrosis factor (TNF) in the presence of serum. However, treatment of the cells with metabolic inhibitors (e.g., cyclohexamide) or by incubation in serum-free medium induces TNF cytotoxicity. The cells die by apoptosis, although some of the molecular markers of cell death paint a mixed necrosis/apoptosis picture. In any case, IGF-I is fully protective against TNF cell killing. Ethanol has no effect on TNF-induced cell killing in the absence of IGF-I, but reverses the protective action of IGF-I.

The inhibition by ethanol of the trophic actions of IGF-I has been examined in rat cerebellar granule cell cultures (Zhang et al. 1998*a*, 1998*b*). Granule cell cultures obtained from 7-day-old pups can be maintained in the presence of depolarizing concentrations of potassium (25 mM: "HK"). The cells undergo apoptosis when the medium is changed to low potassium (5 mM: "LK"). IGF-I supports granule cell viability in LK. The protective action of IGF-I is dependent on PI3K activity, since cell killing in the presence of IGF-I was restored by addition of the PI3K inhibitor LY293002. By contrast, the MAP kinase inhibitor PD 98059 had no effect on IGF-I-mediated neurotrophism. Additional IGF-I signaling mediators may also be involved in neurotrophism, because the PKC inhibitor chelerythrine chloride and the PKA inhibitor KT5720 were also partially effective in reversing IGF-I neuroprotection.

Ethanol is not toxic for granule cells under HK conditions, nor does it accentuate apoptosis in LK. However, ethanol reversed the protective actions of IGF-I in LK. The inhibition of IGF-I protection by ethanol corresponds to a marked reduction in IRS-1 phosphorylation and PI3K activation.

The functional effect of ethanol on IGF-I trophic action in cerebellar granule cells is similar to its effect on the *N*-methyl-D-aspartate (NMDA) receptor. NMDA supports granule cell viability in LK, an effect that is reversed by ethanol (Zhang et al. 1998*b*). The mechanism by which NMDA exerts its neurotrophic effect has recently come under increased scrutiny. Certainly, NMDA-induced calcium influx is a critical factor in neurotrophism, since ethyleneglycoltetraacetic acid (EGTA) completely reverses neuroprotection (Zhang et al. 1998*a*). It is likely that calcium promotes survival, at least in part, via the activation of calmodulin-dependent kinases (Yano et al. 1998; Egea et al. 1999).

In view of the functional similarities of NMDA and IGF-I, we examined whether there is cross-talk between the two signaling systems (Zhang et al. 1998*b*). NMDA was found to phosphorylate IRS-1 and activate PI3K. Ethanol reversed these activities of NMDA, as expected in view of ethanol's action at the level of the NMDA receptor. The dependence of PI3K for NMDA-induced survival was evidenced by its reversal with the PI3K inhibitor LY293002.

At least one mechanism by which NMDA induces IRS-1 phosphorylation has been determined. NMDA induces the secretion of brain-derived neurotrophic factor (BDNF) (Marini et al. 1998). The activation of the BDNF ligand TrkB induces PI3K activation (Yamada et al. 1997; Qiu et al. 1998) (figure 2). Whether NMDA similarly causes IGF-I secretion remains to be determined.

POSSIBLE MECHANISM OF ETHANOL INHIBITION OF IGF-IR

In cell culture models studied to date, the inhibition of IGF-IR activation leads to a comparable reduction in the individual components of downstream signaling. The generalized suppression of these pathways leads to inhibited cell proliferation and sensitization to pro-apoptotic influences. As for all cell culture models, the interpretation of these findings respective to the in vivo situation must be approached cautiously. Importantly, the study of IGF-IR dynamics requires carefully defined media conditions, generally in the absence of serum. Even high concentrations of ethanol are only weak inhibitors of in vitro cell proliferation in the presence of a full complement of growth factors and cytokines in serum. The presence of serum or the actual in vivo situation immeasurably complicates the analysis of specific signaling components due to variable receptor expression, extensive cross-talk, and the cell-specific context of downstream signaling mediators. Only by isolating specific receptor function can ethanol sensitivity be rigorously determined.

Despite these issues, ample progress has been made in our understanding of the in vivo role of the IGF-I/IGF-IR signaling axis. Targeted knockout of the IGF-I and IGF-IR genes leads to profound growth suppression (< 50 percent normal weight), and the fetuses do not survive birth (reviewed in Rubin and Baserga 1995). However, fibroblasts cultured from these embryos (R^- cells) grow in the presence of serum, although at a slower rate than fibroblasts obtained from wild-type littermates (indicating that the IGF-IR is not a strict requirement for optimal cell growth). The autocrine growth of many tumor cell types can be inhibited by incubation with antisense oligodeoxynucleotides. Conversely, body growth generally corresponds to IGF-I levels, and IGF-I transgenic mice are larger than their controls.

The IGF-IR is ubiquitously expressed, and normal serum levels of IGF-I are in a range that supports maximal cell growth in vitro. It is apparent therefore that the activity of the IGF-IR is regulated by factors additional to ligand concentrations (indeed, an issue common to cell surface receptors in general). In the case of IGF-I, a family of binding proteins regulates IGF-I bioavailability (Sara and Hall 1990). However, additional factors must exist at the level of the IGF-IR. For example, IGF-I is not mitogenic for serum-starved 3T3 cells unless they are rendered competent by addition of other growth factors (e.g., PDGF or EGF). PDGF may induce IGF-I sensitivity by induction of IGF-IR expression. On the other

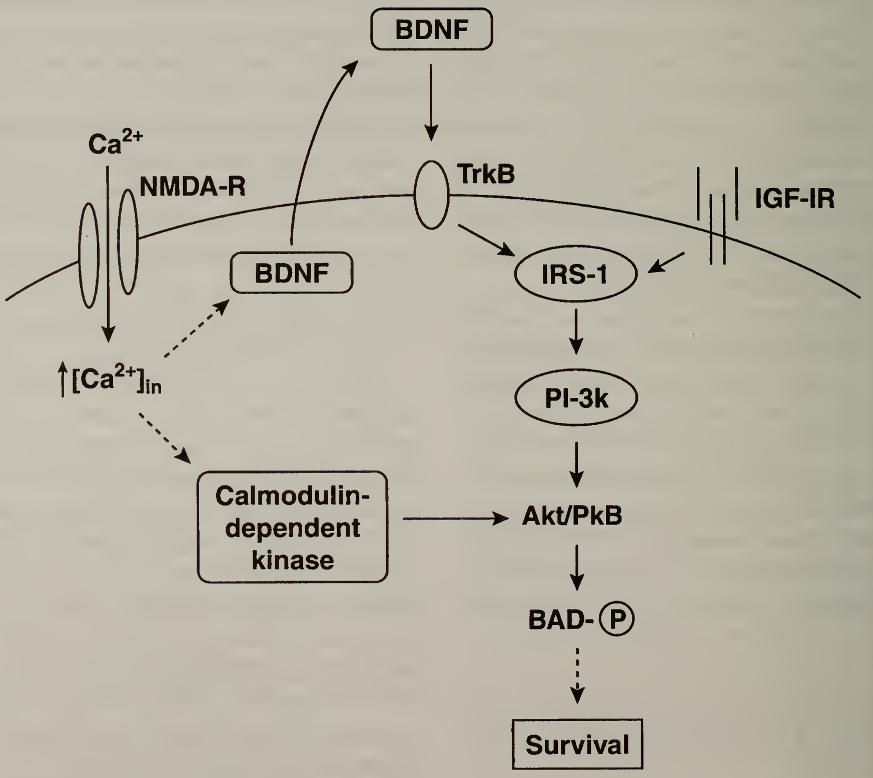

Figure 2. Role of insulin-like growth factor I (IGF-I) and *N*-methyl-D-aspartate (NMDA) receptors in cerebellar granule cell survival. BDNF = brain-derived neurotrophic factor; IGF-IR = IGF-I receptor; IRS-1 = insulin receptor substrate 1; NMDA-R = NMDA receptor; PI-3k = phosphatidylinositol 3-kinase; PkB = protein kinase B; BAD-P = phosphorylated BAD.

hand, IGF-I alone is mitogenic in cells that overexpress the IGF-IR. It has been suggested in this regard that IGF-I-dependent mitogenicity is achieved only at a threshold level of IGF-IR expression (Baserga and Rubin 1993). For example, the temporal and geographic regulation of the IGF-IR is tightly controlled during developing brain. Despite these observations, it is likely that additional intracellular factors influence the functional activity of the IGF-IR, either by direct intracellular β-chain modification (i.e., phosphorylation) or by alterations in the level of mediators that are directly bound and phosphorylated by the IGF-IR.

The intracellular regulatory features of IGF-IR activity undoubtedly have an impact on their sensitivity to ethanol. The specific mechanism by which ethanol interferes with IGF-IR tyrosine autophosphorylation is not known. Ethanol has no effect on ligand binding (Resnicoff et al. 1993). The inhibitory action of ethanol on immunoprecipitated receptors suggests that ethanol directly interacts with the beta subunit tyrosine kinase domain that mediates autophosphorylation. However, recent observations suggest that the sensitivity of the IGF-IR to ethanol is less than straightforward. In fact, several observations suggest that there may be ethanol-sensitive and ethanol-insensitive states of the receptors that are dependent on intracellular factors:

- Under conditions of high IGF-IR expression (i.e., p6 cells), the inhibition of IGF-IR tyrosine autophosphorylation by ethanol can be overcome by high concentrations of IGF-I, despite unaltered ligand binding. In fact, the autocrine proliferation of BALB/c3T3 cells that overexpress both IGF-I and the IGF-IR (p12 cells) is ethanol insensitive (Resnicoff et al. 1996). This contrasts with wild-type fibroblasts in which high concentrations of IGF-I do not overcome the antiproliferative action of ethanol. It appears therefore that high receptor expression can overcome the ethanol effect. The interpretation of this observation is problematic using intact cells alone. For one thing, under conditions of submaximal IGF-IR phosphorylation, it is not clear whether a subset of ethanol-sensitive receptors are affected or if all receptors are equally hypophosphorylated. Furthermore, in intact cells, the dynamics of IGF-IR activation are complicated by multiple events including transphosphorylation, clustering and internalization, and feedback dephosphorylation by phosphatases.
- We have observed that subclones of the p6 cell line display variable sensitivity to ethanol, and some subclones are completely ethanol insensitive (Seiler et al. unpublished data). It has even been reported that ethanol can stimulate IGF-I dependent DNA synthesis in a 3T3 fibroblast subclone (Tomono and Kiss 1995). In these studies, ethanol enhanced [^{3}H]thymidine incorporation. However, a stimulatory effect of ethanol on cell proliferation was not reported. In preliminary studies, we have noted that ethanol sensitivity diminishes as the cells adopt a transformed phenotype. It will be of interest to examine

whether the factors that activate the IGF-IR in transformed cells similarly modulate ethanol sensitivity.
- In chicken embryo retinal cultures, ethanol has no effect on IGF-IR tyrosine autophosphorylation (Seiler et al. unpublished data).
- Ample precedent exists for a role of intracellular factors that alter the sensitivity of receptors to ethanol. Protein phosphorylation and other factors (Diamond and Gordon 1997) modulate the ethanol sensitivity of gamma-aminobutyric acid (GABA) and NMDA receptors. Charness and colleagues recently characterized ethanol-sensitive and -insensitive fibroblast cell lines expressing the immunoglobulin neural cell adhesion molecule (N-CAM) L1 (Wilkemeyer and Charness 1998). The biological relevance of this finding was highlighted by the correspondence of ethanol sensitivity with a similar inhibition by Fab fragments prepared from anti-L1 polyclonal antisera.

In summary, the specific mechanism by which ethanol variably inhibits IGF-IR function remains to be determined. Although some in vitro data indicate a direct interaction of ethanol with the receptor, other factors clearly contribute to the overall issue of ethanol sensitivity.

IMPLICATIONS FOR ALCOHOL-INDUCED DISEASE

Since tissue damage is limited to a subset of heavy alcohol drinkers, it is unlikely that alcohol exerts a general antiproliferative or pro-apoptotic effect in an otherwise healthy individual. In view of the preceding discussion, we offer the hypothesis that the inhibitory action of ethanol on IGF-IR function occurs during periods of enhanced IGF-I dependence for cell growth and survival. It is possible that the intracellular factors that induce IGF-I sensitivity in general may similarly regulate the response of the IGF-IR to ethanol. Importantly, the teratogenicity associated with fetal alcohol syndrome is associated with alcohol ingestion during critical periods of gestation. Another example may be found in the regenerating liver where IGF-IR is preferentially expressed and may play a role in mitogenesis. A reasonable question is whether IGF-IR or other ethanol-sensitive receptors are important for these developmental and reparative transitions.

The interaction of ethanol with growth factor regulatory pathways is clearly not restricted to the IGF-IR. Under selected conditions and different cell types, ethanol has been demonstrated to inhibit signaling by EGF (Saso et al. 1997), insulin (Banerjee et al. 1998), and fibroblast growth factor and PDGF (Luo and Miller 1997*a*). The complicated interplay of ethanol with these pathways was highlighted, for example, by Miller and colleagues, who demonstrated that ethanol can inhibit the growth of several human neuroblastoma cell lines in response to assorted growth factors, including IGF-I (Luo and Miller 1997*a*). However, as detailed above, the IGF-IR may be contributing to mitogenesis in the response of neuronal cells to other growth factors, and further studies would be needed to assess the impact of ethanol on these processes.

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Chapter 8

Ethanol and NF-κB Signaling in Immune Cells

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Key words: ethanol; cell signaling; cell-mediated immunity; receptor ligand binding; biological inhibition; lipopolysaccharide; intracellular transport; laboratory study

Although it is known that chronic alcohol use often adversely affects various organ systems, resulting in cirrhosis of the liver, cardiomyopathy, or immunosuppression, the clinical consequences of acute and moderate alcohol use are yet to be delineated (National Institute on Alcohol Abuse and Alcoholism [NIAAA] 1993). In general, consumption of moderate amounts of alcohol has been associated with both beneficial and harmful effects on health in humans. Decreased mortality and cardiovascular morbidity was found in individuals consuming one to two drinks a day in epidemiologic studies (Goldberg et al. 1995; Kannel and Ellison 1996; Wilt et al. 1996*b*). Indeed, emerging data suggest a protective effect of acute, moderate alcohol consumption on cardiovascular morbidity, likely secondary to protective effects of alcohol on development and progression of atherosclerosis (Wilt et al. 1996*a*).

The mechanisms for the protective effects of moderate alcohol use on atherosclerotic heart disease are yet to be understood. Elevation in high-density lipoproteins (HDL), the cardioprotective cholesterol, in humans is one of the potential components (Kannel and Ellison 1996). However, recent evidence suggests that the inflammatory process participating in endothelial damage is key to atherosclerotic plaque formation; thus, it may be an important target for the effect of ethanol (Laman et al. 1997; Zakhari 1997). Inflammatory

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mediator and chemokine induction as a result of endothelial cell and macrophage activation have been identified as key elements in initiation and progression of atherosclerotic plaque formation. Decreased production of the inflammatory cytokine tumor necrosis factor alpha (TNFα) and the chemokine MCP-1 (monocyte chemoattractant peptide-1) was reported both in human monocytes after acute alcohol treatment and in murine macrophages after binge drinking (Nelson et al. 1989; Verma et al. 1993; Szabo et al. 1996, 1999). Such impaired inflammatory cytokine production has been shown to contribute to decreased host defense and increased susceptibility to infections (Nelson et al. 1992). Consequently, acute alcohol use impairs the capacity of the immune system in responding to infectious stimuli. The cellular mechanisms by which acute ethanol inhibits the production of inflammatory cytokines and chemokines are yet to be fully explored. One of the common regulatory elements in the promoter region of the inflammatory cytokine and chemokine genes is the nuclear regulatory factor κB (NF-κB) responsive element (Ahakhov et al. 1990; Collart et al. 1990; Bauerle and Henkel 1994). Thus, we hypothesized that ethanol may modulate NF-κB activation in monocytes/macrophages as a common target to modulate inflammation-related gene activation.

The nuclear regulatory protein NF-κB/Rel is a family of nuclear regulatory factors typically residing in the cytoplasm as dimers anchored to the inhibitory κB molecules, IκBα and IκBβ (Beg and Baldwin 1993; Kopp and Gosh 1994; Bauerle and Henkel 1994). Upon cellular activation with various agents, including bacterial stimulation, lipopolysaccharide (LPS), and superantigens, IκB is rapidly phosphorylated and degraded via the ubiquitin-proteasome pathway, allowing nuclear translocation of the NF-κB dimer (May and Gosh 1998). The typical transactivating NF-κB/Rel dimer is the p65/p50 heterodimer, but other dimers, like p50/p50 homodimer, contain nuclear binding domain without possessing the transactivating domain. Acute ethanol treatment of Kupffer cells was shown to decrease LPS-induced NF-κB activation (Fox et al. 1996). We have previously reported that acute ethanol treatment increases NF-κB nuclear binding in blood monocytes; however, the ethanol-induced complex was different from the NF-κB complex induced by LPS stimulation (Mandrekar et al. 1997). Here, we show that acute in vitro ethanol treatment prevents LPS-induced NF-κB activation in human monocytes. Our studies further revealed that ethanol has multiple target sites for inhibition of NF-κB, including IκBα phosphorylation, preferential p50/p50 homodimer induction, and, likely, induction of the glucocorticoid receptor.

METHODS

Reagents

We obtained Iscove's modified Dulbecco's medium, RPMI 1640, from Gibco (Gaithersburg, MD) and fetal bovine serum (FBS) from Atlanta Biologicals (Norcross, CA). Endotoxin contamination was less than 8 pg/mL in the culture media or FBS. LPS (*Escherichia coli* strain 0111:B4) was obtained from Difco Laboratories (Detroit, MI). NF-κB oligonucleotide (sequence: 5'AGTTGAGGGGACTTTCCCAGGC3') was obtained from Promega (Madison, WI). Protease inhibitors, including phenylmethyl sulfonyl fluoride (PMSF), aprotinin, antipain, and leupeptin, were from Sigma Chemical Company (St. Louis, MO).

BLOOD DONORS

Healthy nonalcoholic individuals, ages 18–60, females and males, donated 120 mL peripheral blood per experiment. Blood was obtained by venipuncture and anticoagulated with heparin. Blood donors had no previous alcohol abuse history, consumed less than six drinks per week, and consumed no alcohol for at least 48 hours before blood donation.

MONOCYTE SEPARATION AND STIMULATION

Monocytes from human peripheral blood were isolated by selective adherence from Ficoll-Hypaque purified mononuclear cell preparations as previously described (Szabo et al. 1995). Briefly, Ficoll-Hypaque density-separated mononuclear cells were washed in ice-cold Hank's balanced salt solution supplemented with 5 percent FBS and adhered to microexudate-treated flasks at 1 × 10^7 cells/flask in RPMI containing 15 percent FBS. Nonadherent cells were removed after 2-hour adherence by gentle washing with warm RPMI, and then adherent cells were collected by vigorous shaking with 4 mM ethylenediaminetetraacetic acid (EDTA). This separation provides greater than 94 percent monocyte purity. Monocytes were collected and plated in six-well plates for stimulation at 1 × 10^7 cells/group for nuclear and cytoplasmic extractions and 3 × 10^6 cells/group for bioassays. Cells were stimulated with *E. coli*–derived LPS (1 µg/mL), 25mM ethanol, and the combination of LPS and ethanol added together. The 25 mM in vitro ethanol concentration approximates a 0.1 g/dL blood alcohol level, which is easily achieved in vivo after moderate drinking (three to four drinks). Cell viability was not affected by ethanol or LPS treatment.

PREPARATION OF NUCLEAR AND CYTOPLASMIC EXTRACTS

Cells with or without 1-hour stimulation at 37 °C were extracted by the modified method of Dignam and colleagues (1983). At the end of the stimulation period, cells were washed in ice-cold phosphate-buffered saline three times and scraped in cold buffer A (10 mM Tris-HCl [pH 7.8], 5 mM $MgCl_2$, 10 mM NaCl, 0.5 mM dithiothreitol [DTT], 0.3 M sucrose, 0.5 mM PMSF, 0.1 mM EDTA, and 1 mg/mL protease inihibitors such as aprotinin, antipain, and leupeptin). Cells were then lysed using a glass Dounce homogenizer on ice with 15–20 strokes. The lysate was centrifuged at 12,000 g (gravity) for 30 seconds to pellet the nuclei, and the supernatant was stored at –80 °C as the cytoplasmic extract. The nuclear pellet was then resuspended in ice-cold buffer B (20 mM Tris-HCl [pH 7.8], 5 mM $MgCl_2$, 300 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, and 25 percent glycerol). All tubes were placed on a rotator in 4 °C for 30 minutes. The lysate was then centrifuged at 12,000 g for 15 minutes, and the supernatant was stored at –80 °C as the nuclear extract. Protein content was estimated in both the cytoplasmic and nuclear extract by the Bio-Rad Dye Reagent Assay (Bio-Rad, Hercules, CA).

ELECTROPHORETIC GEL MOBILITY SHIFT ASSAY

A consensus double-stranded NF-κB oligonucleotide (Promega; sequence: 5'AGTTGAGGGGACTTTCCCAGGC3') was used for electromobility shift assays (EMSA). End-labeling was accomplished by treatment with T4 kinase in the presence of ^{32}P-adenosine triphosphate. Labeled oligonucleotides were purified on a polyacrylamide copolymer column (Bio-Rad). Five micrograms of nuclear protein was added to a binding reaction mixture containing 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 20 percent glycerol, 20 µg/mL bovine serum albumin, 2 µg poly(dI-dC), and 30,000 cpm of ^{32}P-labeled NF-κB oligonucleotide. Samples were incubated at room temperature for 30 minutes. For supershift assays, 1 µL of the antibody was added to the reaction after the 30 minutes and then further incubated for 30 minutes. All reactions were run on a 5 percent polyacrylamide gel, and the dried gel was exposed to an x-ray film at –80 °C overnight. Anti-human p50 antibody for supershifts was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit antisera raised against the N-terminus region of human p65 was generously provided by Dr. Nancy Rice (ABL-Basic Research Programs, Frederick, MD). Cold competition was done by adding a twentyfold excess of specific unlabeled double-stranded probe to the reaction mixture.

WESTERN BLOTS

Ten-microgram aliquots of nuclear or cytoplasmic proteins were mixed with an equal volume of 2X sample buffer (containing 0.1 percent sodium dodecyl sulfate [SDS] and 2-mercaptoethanol) and boiled for 5 minutes. Proteins were separated on 10 percent SDS-polyacrylamide gel and electroblotted onto nitrocellulose membranes. Nonspecific binding was blocked by soaking the membranes in Tris-buffered saline (TBS)/1 percent nonfat dried milk/0.1 percent Tween-20 for 30 minutes at room temperature. Immunoreactive proteins were detected by incubating the blots with diluted p50 and p65 antibodies at 4 °C overnight. After washing the blots in TBS/0.1 percent Tween-20, the filters were incubated with horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin G (IgG) (Amersham, Arlington Heights, IL). Blots were then washed again in TBS/Tween-20 and developed by the use of an enhancer chemiluminescence assay reagent from Amersham. NF-κB p50 (SC-114) affinity-purified rabbit polyclonal antibody was obtained from Santa Cruz Biotechnology and rabbit polyclonal antibody against the C-terminal peptide of p65 (SA-171) from Biomol (Plymouth Meeting, PA). Anti-IκBα antibody was a rabbit polyclonal IgG1 against the carboxy terminal epitope of human IκBα (SC-371) and antiglucocorticoid receptor antibody, and affinity purified rabbit polyclonal antibody against a peptide corresponding to the amino-terminus region of the human glucocorticoid receptor was from Santa Cruz Biotechnology.

RESULTS

ACUTE ETHANOL TREATMENT INHIBITS LPS-INDUCED NF-κB NUCLEAR BINDING IN HUMAN MONOCYTES AND MURINE MACROPHAGES

We previously reported that acute ethanol treatment of human monocytes results in inhibition of LPS or superantigen-induced interleukin-1β (IL-1β) and TNFα production at both the bioactive protein and messenger RNA levels (Szabo et al. 1996). We also found that exposure of monocytes to alcohol for even 1 hour prevents maximal TNFα induction by LPS, suggesting that ethanol affects early signaling events in TNFα induction (Mandrekar et al. 1997). To evaluate the effect of acute ethanol on NF-κB activation in immune cells, blood monocytes from nonalcoholic individuals were exposed ex vivo to a physiologically relevant dose of alcohol (25 mM, which is equivalent to 0.1 g/dL blood alcohol level) in combination with LPS, then NF-κB specific binding of nuclear extracts was tested in EMSA. LPS has previously been shown in multiple studies to induce NF-κB activation in monocytes/macrophages. As illustrated in figure 1A, unstimulated blood monocytes show a minimal NF-κB binding, which is likely due to adherence-induced activation of NF-κB (Ahakhov et al. 1990; Collart et al. 1990; Bauerle and Henkel 1994). Whereas ethanol alone resulted in a modest increase, LPS stimulation was a very potent inducer of NF-κB nuclear binding in human monocytes. In contrast, the LPS-induced NF-κB binding was substantially reduced in the presence of ethanol.

To test whether the inhibition of NF-κB activation by ethanol is specific for human monocytes or occurs in other monocytic cells as well, we tested the murine P388D1 macrophage cell line. Here we found that the superantigen staphylococcal enterotoxin B (SEB) is a potent inducer of NF-κB in murine macrophages (figure 1B). Similar to that seen in blood monocytes, acute ethanol treatment prevented NF-κB activation by SEB in adherent murine macrophages. These results suggest that acute ethanol exposure can prevent NF-κB activation induced by either Gram-negative bacteria (LPS) or Gram-positive microorganism–derived (SEB) stimuli in monocytic cells.

ACUTE ETHANOL REGULATES CYTOPLASMIC IκBα IN MONOCYTIC CELLS

The NF-κB complex in the cytosol is stabilized via its interaction with the IκBα or IκBβ molecules (Beg and Baldwin 1993). One of the initial steps in activation of NF-κB is phosphorylation and degradation of IκBα, the predominant IκB molecule in monocytic cells. Thus, IκBα levels from ethanol and/or LPS-stimulated monocytes were evaluated by reacting cytoplasmic extracts with anti-IκBα antibody in Western blot experiments. Unstimulated monocytes contained substantial cytoplasmic IκBα levels (figure 2), consistent with little

NF-κB activity seen in the nuclear extracts (see figure 1). LPS stimulation resulted in a rapid degradation of IκBα and almost complete reaccumulation was seen by 3 hours after stimulation (figure 2). Such kinetics of LPS-induced IκBα regulation is consistent with previous reports. In contrast, acute ethanol treatment alone did not change cytoplasmic IκBα levels over time. Unlike with ethanol stimulation alone, decreased IκBα levels were seen even after addition of ethanol with LPS both at 60 minutes (data not shown) and at 120 minutes (figure 2) after stimulation. These results suggest that ethanol decreases NF-κB nuclear binding without preventing LPS-induced degradation of IκBα in monocytes/macrophages.

Since IκBα degradation is typically preceded by its phosphorylation, we tested the effect of ethanol on the levels of phosphorylated IκBα. Phospho-IκBα (P-IκBα) was evaluated in the cytoplasmic extracts of LPS and/or ethanol-treated blood monocytes at 0–240 minutes after stimulation. There was only minimal P-IκBα seen in unstimulated monocytes (figure 3). Ethanol treatment alone did not change P-IκBα levels over unstimulated monocytes, consistent with no change

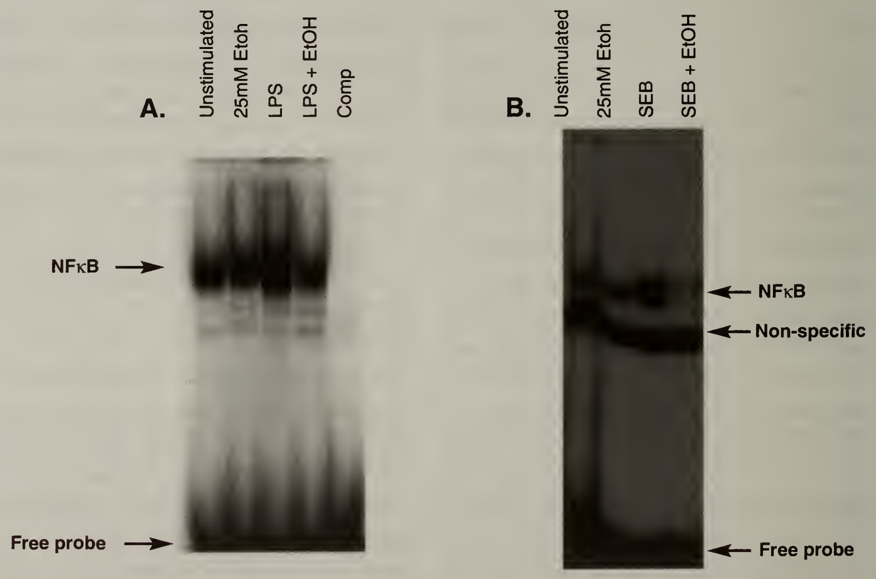

Figure 1. Decreased NF-κB activation in monocytic cells treated with acute ethanol (EtOH). (A) Monocytes were stimulated with EtOH, 1 µg/mL lipopolysaccharide (LPS), or their combination for 1 hour. (B) P388D1 murine macrophages were stimulated with EtOH, 1 µg/mL staphylococcal enterotoxin B (SEB), or their combination for 1 hour. For both cell types nuclear extracts were then prepared and gel shift analysis performed as described in the Methods section. Representative electromobility shift assay of three separate experiments is shown using ^{32}P-NF-κB consensus oligonucleotide. A twentyfold excess of the unlabeled NF-κB fragment was included as a cold competitor (Comp).

in total IκBα levels seen in figure 2. As expected, LPS stimulation alone increased P-IκBα levels, and the increase was seen as early as 1 minute and lasted for at least 60 minutes after stimulation (figure 3). In contrast to P-IκBα activation by LPS alone, combined stimulation with ethanol and LPS resulted in no increase in monocyte P-IκBα levels at any time points, suggesting that ethanol prevents LPS-induced IκBα phosphorylation. These results suggest that acute ethanol is likely to target IκBα phosphorylation as a mechanism for inhibiting NF-κB activation.

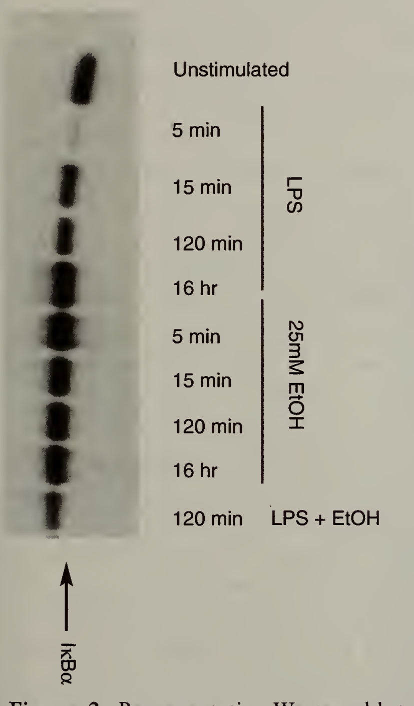

Figure 2. Representative Western blot analysis demonstrating the effect of ethanol (EtOH) on the total IκBα levels. Cytoplasmic extracts (20 µg/group) of monocytes stimulated with 1 µg/mL lipopolysaccharide (LPS) with or without 25mM ethanol for the indicated length of time were subjected to immunoblotting using the anti-IκBα antibody as described in the Methods section.

Ethanol Induces Nuclear Proteins With NF-κB Inhibitory Activity in Macrophages

Decreased IκBα in LPS+ethanol–stimulated monocytes suggests that NF-κB is being dissociated from the NF-κB/IκB complex in the cytoplasm, yet nuclear binding of NF-κB is reduced. Decreased NF-κB DNA binding can be due to preferential induction of alternate NF-κB dimers or to inhibition of NF-κB DNA binding by other nuclear proteins. To further investigate the mechanisms for ethanol-induced decrease in NF-κB nuclear binding, we modified EMSA to conditions that separate the various NF-κB dimers. Figure 4 shows that unstimulated monocytes predominantly contained a lower molecular weight NF-κB complex, complex II, while LPS induced an additional, higher molecular weight complex, complex I. Addition of ethanol to LPS stimulation resulted in a selective elimination of the LPS-induced NF-κB complex (complex I) in monocytes.

Based on our and other previous studies, complex I represents NF-κB binding of the p65/p50 heterodimer and complex II represents NF-κB binding of the p50/p50 homodimer. Ethanol alone increased the nuclear binding of complex II (p50/p50 homodimer) compared with unstimulated monocytes. The specificity of complexes I and II was confirmed in

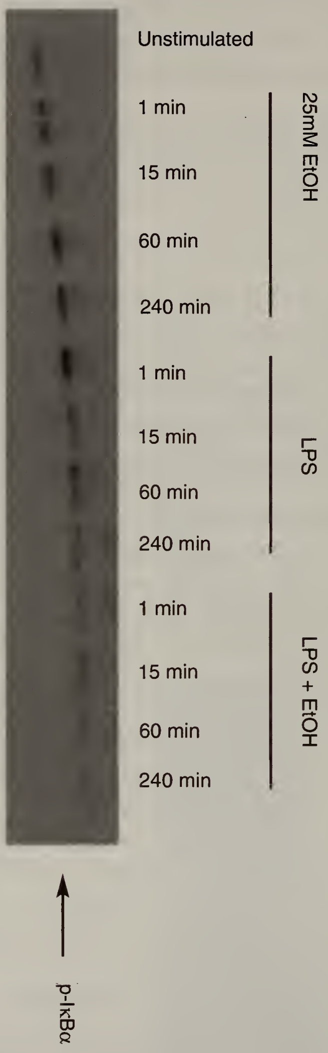

Figure 3. Decreased phospho-IκB levels in ethanol-treated monocytes. Cytoplasmic extracts (20 μg/group) of monocytes stimulated with 1 μg/mL lipopolysaccharide (LPS) with or without 25mM ethanol for the indicated length of time were subjected to immunoblotting using the phospho-specific anti-IκBα (p-IκBα) (serine 32) antibody as described in the Methods section.

supershift experiments in which anti-p50 antibody supershifted all NF-κB complexes in unstimulated, LPS-stimulated, or ethanol-stimulated monocytes (figure 5). In contrast, anti-p65 antibody supershifted only complex I (p65/p50 heterodimer), which was induced in LPS-stimulated (and not in LPS+ethanol–stimulated) monocytes, confirming that ethanol prevents LPS-induced nuclear binding of p65/p50 heterodimer (figure 6). Consequently, ethanol appears to have a dual effect on NF-κB binding: it inhibits nuclear binding of LPS-induced p65/p50 heterodimer, with a concomitant increase in the binding of the p50/p50 homodimer. Such an effect of ethanol is likely to be biologically significant, considering that the p50/p50 homodimers have the DNA binding but no DNA transactivation domain and thus can bind but not transactivate the NF-κB nuclear regulatory element (Ziegler-Heitbrock et al. 1994; Muller et al. 1995).

Finally, other transcription factors, particularly the glucocorticoid receptor, have been shown to selectively inhibit DNA binding of p65, a phenomenon similar to that seen in ethanol-treated monocytes (Ray and Prefontaine 1994; Heck et al. 1997; Wissink et al. 1997). Thus, the effect of ethanol was tested on glucocorticoid receptor activation and nuclear translocation. Ethanol consumption itself has been shown to result in increased glucocorticoid induction. Here, we quantified the nuclear levels of glucocorticoid receptor in the macrophage cell line P388D1 by Western blots (figure 7). There was no detectable glucocorticoid receptor expression in the nuclear extracts of unstimulated macrophages. After stimulation with either LPS or ethanol alone, substantial levels of nuclear glucocorticoid receptor were seen. Moreover, combined stimulation of monocytes with LPS+ethanol resulted in an even greater increase in nuclear glucocorticoid receptor levels. These results show that ethanol and LPS potentiate each other's effect in increasing nuclear glucocorticoid receptor levels while resulting in decreased NF-κB activity.

DISCUSSION

Investigation of the effect of acute ethanol on monocyte NF-κB activation in our experiments revealed that ethanol prevents LPS-induced induction of the NF-κB p65/p50 heterodimer. Our results suggest that the mechanisms by which ethanol downregulates NF-κB activation in monocytic cells is likely to involve multiple elements: decreased IκBα phosphorylation, preferential induction of p50/p50 homodimer in the presence

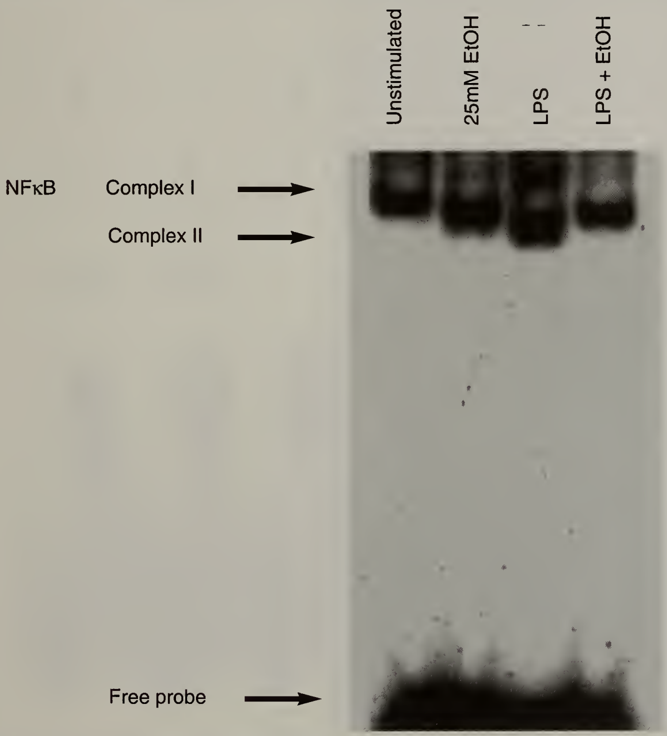

Figure 4. Prevention of lipopolysaccharide (LPS)-induced NF-κB complex activation with acute ethanol (EtOH) treatment. Adherence-isolated human monocytes were stimulated for 1 hour with 25 mM ethanol, 1 μg/mL LPS, or their combination, then nuclear extracts were prepared. Equal amounts of nuclear protein were loaded for electromobility shift assays as described in the Methods section.

of ethanol, and increased nuclear glucocorticoid receptor levels. Each of these mechanisms is likely to contribute to decreased LPS-induced NF-κB binding in ethanol-exposed monocytes and macrophages.

The biological significance of decreased NF-κB activation in acute ethanol–exposed monocytes and macrophages in response to conventional inducers of NF-κB such as LPS and SEB is potentially very broad. NF-κB-driven genes include most of the inflammation-related mediators, cytokines, and chemokines, which play a pivotal role in host defense during invasion by microbial pathogens (Ahakhov et al. 1990; Collart et al. 1990; Bauerle and Henkel 1994; Kopp and Gosh 1994). Furthermore, inflammatory cytokines as well as chemokines play a pivotal role in progression of chronic diseases that have inflammatory components, such as atherosclerosis. Monocyte-derived TNFα and MCP-1 were shown as key mediators inducing endothelial cell injury in atherosclerotic plaque formation (Laman et al. 1997). Considering the cardioprotective effect of moderate

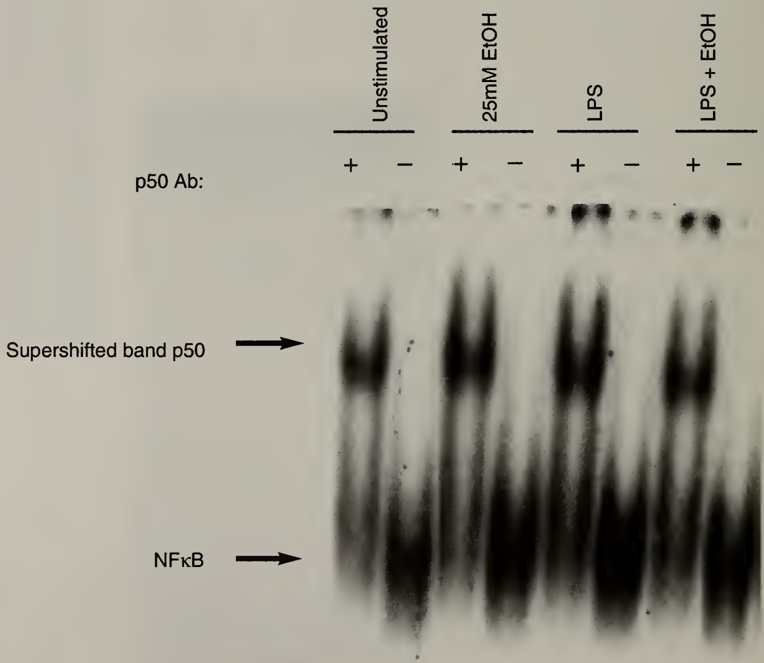

Figure 5. p50 Supershift analysis of NF-κB in ethanol (EtOH)-treated monocytes. Nuclear extracts from monocytes stimulated for 1 hour with 1 µg/mL lipopolysaccharide (LPS) with or without 25mM EtOH were subjected to gel shift analysis. All reactions were admixed with rabbit anti-p50 antibody (Ab) (2 µg) after addition of ^{32}P-NF-κB oligonucleotide and incubated for 30 minutes. Shifted bands were detected by their retarded mobility.

alcohol consumption, we speculate that inhibition of NF-κB may be a common mechanism by which ethanol decreases inflammatory mediator induction, thereby potentially reducing progression of atherosclerosis. Considering that alcohol use is cardioprotective only at moderate doses (one to two drinks per day), which result in transient elevation of blood alcohol

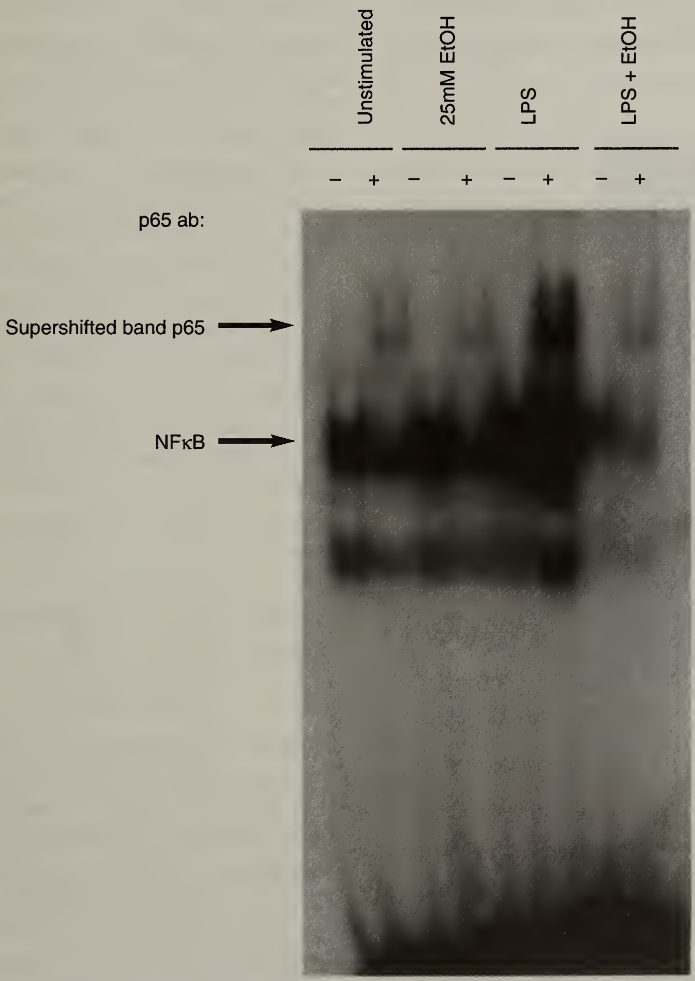

Figure 6. p65 Supershift analysis of NF-κB in ethanol (EtOH)-treated monocytes. Nuclear extracts from monocytes stimulated for 1 hour with 1 µg/mL lipopolysaccharide (LPS) with or without 25mM EtOH were subjected to gel shift analysis. All reactions were admixed with rabbit anti-human p65 antisera after addition of ^{32}P-NF-κB oligonucleotide and incubated for 30 minutes. Shifted bands were detected by their retarded mobility.

levels, acute ethanol treatment in our experiments is likely to be a good representation of the in vivo effect of moderate alcohol use. Inhibition of NF-κB by ethanol is likely to mediate the down-regulation of monocyte TNFα, IL-1β, and MCP-1 production seen at the protein levels. Such impairment of inflammatory mediator induction in response to a subsequent bacterial challenge can certainly also contribute to decreased host defense after acute alcohol use.

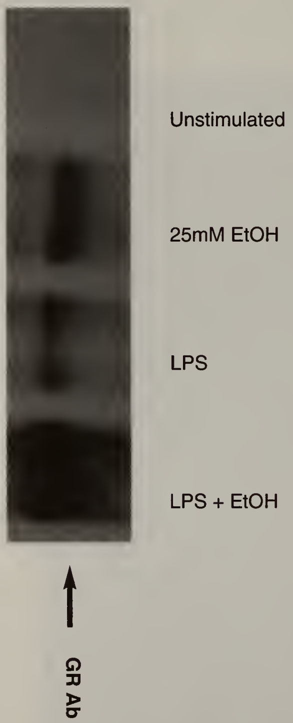

Figure 7. Increased nuclear levels of glucocorticoid receptor (GR) with ethanol (EtOH) treatment. Monocytes were stimulated with 25 mM EtOH, 1 μg/mL lipopolysaccharide (LPS), or their combination, and then nuclear extracts were prepared. Equal amounts of nuclear protein (10 μg/group) were subjected to immunoblotting using the anti-GR antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

We found that ethanol inhibits NF-κB nuclear binding in monocytic cells, whether activated by LPS or the superantigen SEB. LPS typically binds and signals through CD14, while the receptors for SEB on monocytes are the major histocompatibility complex (MHC) class II molecules (See et al. 1992; Labeta et al. 1993; Szabo et al. 1995). Consequently, ethanol is likely to act downstream from receptor activation to prevent NF-κB activation.

Regulation of NF-κB nuclear binding by ethanol in monocytes appears to involve multiple target sites. Our data demonstrated that ethanol decreases NF-κB nuclear binding without preventing IκBα degradation in the cytoplasm. In search of an explanation for decreased NF-κB nuclear binding with concomitantly reduced cytoplasmic IκBα in LPS-induced monocytes, we tested the effect of ethanol on the levels of phosphorylated IκBα. Unexpectedly, LPS-induced increase in phosphorylated IκBα was prevented by acute ethanol treatment of monocytes. By convention, degradation of IκBα is triggered by its phosphorylation (Gosh and Baltimore 1990). Yet, ethanol+LPS–exposed monocytes showed no change in IκBα compared with LPS-treated cells, while phosphorylated IκBα levels remained low. This observation needs further investigation. A plausible explanation would be that ethanol uses an alternate trigger for IκBα degradation that does not require IκBα phosphorylation. Alternatively, phosphorylation only at one site (serine 32), which is recognized by the anti-P-IκBα antibody used in our experiments, may not be sufficient to prevent degradation of IκBα.

One can argue that since ethanol does not prevent IκBα degradation in LPS-stimulated monocytes, NF-κB should be freed to translocate to the nucleus. However, our data consistently show that ethanol prevents LPS-induced NF-κB nuclear binding. Observation of the selective inhibitory effect of ethanol on p65/p50 heterodimer activation and the relative increase in the proportion of p50/p50 homodimers may offer an explanation to this paradigm. Considering that p50 has only the DNA binding and not the the transactivating domain, the relative excess of p50/p50 homodimer in ethanol-treated monocytes is likely to occupy DNA binding sites and potentially prevent p65/p60 heterodimer binding. This would result in decreased activation of NF-κB-dependent genes, reflected by decreased TNFα and IL-β production as seen in ethanol-treated monocytes (Szabo et al. 1995, 1996).

Finally, we propose that ethanol-induced nuclear proteins other than NF-κB/Rel may interfere with p65/p50 binding in monocytes, providing a mechanism for the ethanol-induced decrease in LPS- or SEB-induced NF-κB binding (Ray and Prefontaine 1994; Heck et al. 1997; Wissink et al. 1997). NF-κB is inhibited by glucocorticoids via interaction of the hormone-activated glucocorticoid receptor and NF-κB (Candelhoven et al. 1995). The Rel homology domain of p65 (RelA) was found to associate with the glucocorticoid receptor to repress RelA-mediated activation of intracellular adhesion molecule-1 (ICAM-1) NF-κB site (Candelhoven et al. 1995). Our results show that acute ethanol and LPS stimulation potentiate each other's effect in increasing nuclear glucocorticoid receptor levels. However, in the same monocytes, p65/p50 NF-κB nuclear binding is reduced, raising the possibility that elevated nuclear glucocorticoid receptors may interfere with p65 (RelA) nuclear binding.

In summary, based on our experiments investigating the possible mechanisms involved in acute ethanol–induced inhibition of NF-κB activation by LPS, we propose the following targets for ethanol's intracellular effect: inhibition of LPS-induced IκBα phosphorylation, preferential induction of p50/p50 homodimers, and induction of nuclear glucocorticoid receptors (figure 8). Each one of these mechanisms independently could result in decreased LPS-induced NF-κB nuclear binding as seen in ethanol-exposed monocytes. However, ethanol appears to concomitantly affect all three of these mechanisms in LPS-stimulated monocytes. The biological significance of the multiple sites affected by ethanol in inhibition of NF-κB activation is to be further investigated. There is no obvious common, underlying mechanism to link together decreased phosphorylation, preferential p50/p50 homodimer induction, and increased nuclear glucocorticoid receptor expression based on our experiments or current literature. Thus, ethanol appears to interact with intracellular signaling and regulate its events at multiple levels.

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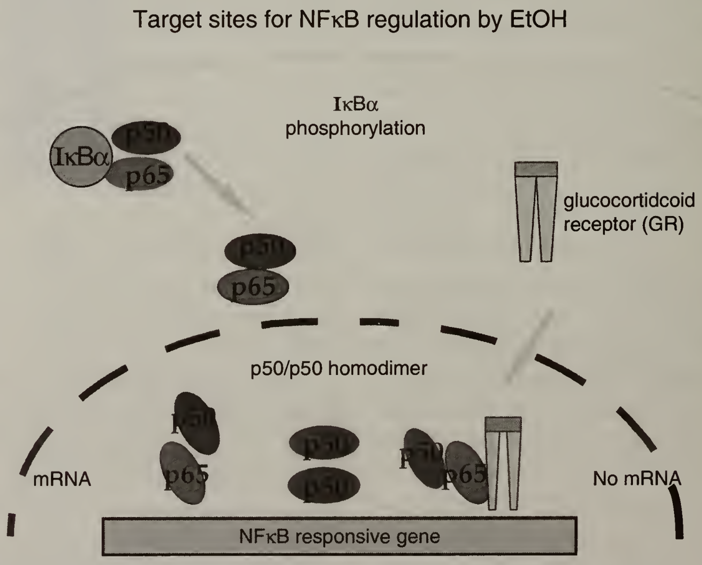

Figure 8. A model for NF-κB regulation by acute ethanol (EtOH) treatment in monocytes. mRNA = messenger RNA.

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ETHANOL AND INTRACELLULAR SIGNALING: GENES AND BEHAVIOR

Chapter 9

Genetic Modification as a Tool for the Study of Behavioral Consequences of Signaling Processes

John C. Crabbe, Ph.D.

Key words: genotype; cell signaling; gene expression; behavior; selective breeding; genetic mapping; AOD (alcohol or other drug) withdrawal syndrome; genetic theory

Genetic modification of experimental organisms, principally the mouse, has become commonplace in neuroscience. There is a rich tradition of genetic studies on which modern methods such as targeted gene replacement are building. In this chapter I discuss some of the more commonly used genetic methods and identify their strengths and limitations.

How genes influence behavior has been a problem of long-standing interest in neuroscience. Complete solution of this problem for any given gene, or for any complex behavior, has eluded neuroscientists to date partly due to the vast range required to encompass DNA and behavior. Behavioral genetics has always been characterized by a willingness to use multiple tools and multiple levels of analysis in behavioral investigation. Some of these approaches, and their strengths and limitations, will be discussed in this chapter. It may be useful to consider first that there are two sources of genetic variation among individuals. *Allelic differences* lead to different gene products, or to gene products with different levels of biological activity. *Gene expression differences* can be allelic, but are more often due to *epistatic* effects of other, interacting genes.

METHODS FOR MODIFYING A GENOTYPE

Selective Breeding

The oldest method in genetic analysis is to modify the genotype indirectly,

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through selective breeding. By mating like with like, livestock have long been improved for agricultural traits. Similarly, since Tolman (1924) and Tryon (1940) selected rats to be "maze bright" or "maze dull," behavioral neuroscientists have recognized the power of arranged matings to produce genetic animal models. In the alcohol abuse area, for example, we have produced lines of Withdrawal Seizure-Prone (WSP) mice that have severe handling-induced convulsions following chronic ethanol vapor exposure. Withdrawal Seizure-Resistant (WSR) lines show virtually no response to chronic ethanol. Several neuropharmacological changes occur differentially in WSP and WSR mice following chronic ethanol, including adaptations in gamma-aminobutyric acid type A ($GABA_A$) and *N*-methyl-D-aspartate (NMDA) receptors, as well as dihydropyridine-sensitive Ca^{++} binding sites. These adaptations could explain the enhanced nervous system excitability characteristic of drug withdrawal (Samson and Harris 1992). A detailed discussion of the methodological aspects of using and interpreting selected lines is presented elsewhere (Crabbe 1999), and some examples drawn from preclinical research in the area of alcohol and other drug abuse have been reviewed (Crabbe and Li 1995).

Selectively bred lines come to differ in allele frequencies for nearly all (and only those) genes affecting the selected trait. They also can differ dramatically in the magnitude of the selected behavior, making them a valuable model for mechanism-oriented research. A limitation of research with selected lines is that the genes affected by selection remain anonymous, and their identity must be inferred indirectly through pharmacological responses and other manipulations. Nonetheless, this has proven to be a very fertile method in behavioral genetics.

CANDIDATE GENE METHODS

Transgenic Over- or Underexpression of a Gene

The advent of the molecular biological revolution has given us more direct means for altering an individual's genotype. If we have a candidate gene for which we suspect an important role in our behavior of interest, it may be possible to create a transgenic mouse. If one inserts a copy of a mouse gene, or of a human gene homologous to that mouse gene, the resulting animal may show signs of overexpression—that is, a superabundance of the gene product. If a targeted gene is altered before reinsertion, the result may be an underexpression of the gene product. Although these methods pioneered the manipulation of candidate genes, difficulty in interpreting the outcomes of such experiments has led most investigators to select the related method of targeted gene deletion. Many of the strengths and weaknesses of these techniques are discussed elsewhere (Wehner and Bowers 1995). However, functional genes can sometimes be inserted by retroviral or adenoviral vectors, and the potential exists to limit gene expression to relevant brain areas, for example, using such vectors.

Targeted Gene Deletion

The preponderance of genetic manipulation under way in neuroscience is using the method of targeted gene deletion. When the substituted DNA is incapable of directing synthesis of functional protein, the gene is successfully "knocked out." The theoretical advantages of such a strategy are fairly obvious, and many intriguing findings have appeared based on studies of the hundreds of knockouts currently extant. For a review of studies of null mutants used to study responses to alcohol and other drugs, see Crabbe and Phillips 1998. However, there are some limitations to the study of knockouts, some well known, and others less obvious but no less important.

Some genes are lethal when deleted. Although some phenotypes can be studied even in embryonic or prenatal animals, behavioral end points require viable postnatal offspring, and some genes cannot be deleted without lethality. Developmental compensation is a major issue for which there is no current, widely applicable solution. If a gene product is important to the organism, it may be assumed that a null mutant will attempt to compensate for its absence through other neurobiological mechanisms. Whether or not such compensations occur in the same neurobiological pathways or systems is entirely unknown. Sometimes the evidence for compensation is unequivocal. For example, Caron knocked out the dopamine transporter gene in mice, and the resulting null mutants displayed an array of alterations in dopamine homeostasis and in other systems (e.g., pituitary) regulated by dopamine (Giros et al. 1996; Gainetdinov et al. 1998).

When conditional knockouts are widely available, it will become possible to effect gene deletion in adults, and/or in specific brain areas. Such subtle refinements of gene targeting are in progress, and their availability will lead to the next major change in genetic methods for neuroscience.

To date, the best source of embryonic stem cells, in which the altered gene constructs are incorporated into embryonic DNA, is from mice of the 129 strain. Most investigators cross the 129-strain embryos with another inbred mouse strain (usually C57BL/6) and then maintain the knockout genotype on this hybrid background of mixed C57BL/6 and 129 alleles. Alternatively, the knockout is gradually backcrossed to either the C57BL/6 or the 129 progenitor strain, which requires 7–10 generations of crosses before the background strain attains 99 percent identity with the inbred progenitor, differing only in the region closely surrounding the transgene. During this transition (and at any time in a hybrid strain maintained on a segregating background), other genes may exert effects that modify the effect of the transgene. These *genetic background effects* are thought to be due to allelic epistasis and can make it very difficult to interpret the results from the knockout experiment. These issues are discussed in more detail elsewhere (Gerlai 1996; Crawley et al. 1997; Phillips et al. 1999).

129 Mice and Background Effects

Recently, scientists have started to refer publicly to 129 mice as a poor choice for the placement of targeted null mutations. These mice are stated to be sluggish and stupid, to have a wide range of other behavioral abnormalities, and to have abnormal brains.

The last point can be traced to findings that in certain 129 strains, a percentage of mice lack the corpus callosum (Livy and Wahlsten 1997), but evidence for the former points is rather thin. The reality is that less is known about the 129 strains than many other, more common inbred strains (e.g., BALB/c, C3H/He, C57BL/6, DBA/2), about which the same vague characterizations of behavioral inadequacy could be made. More importantly, there are many different substrains of 129 (e.g., 129/J, 129/SvJ, 129/ReJ, 129/SvEv-Tac). Although these are closely related (any two 129 substrains are far more closely related than either is to C57BL/6, for example), they are not identical. In a recent paper, substantial polymorphism was demonstrated for microsatellite markers among several 129 substrains, although the level of polymorphism for functional genes was much less (Simpson et al. 1997).

Several recommendations have been made regarding how to deal effectively with the issues of background strain of choice for a knockout and the possibility of epistatic effects of modifier genes (Crawley et al. 1997). In general, choice of a background strain should be made with reference to the expected phenotype. If one's goal is to detect the effect of a knocked-out gene on visual discrimination learning, then the C3H/HeJ strain is a poor choice as a recipient of the mutation, because this strain is known to have retinal degeneration. A second general suggestion is to backcross the genetically modified construct onto exactly the same substrain of 129 that supplied the original embryonic stem cell. This method would nearly completely avoid the interference of modifier genes introduced at the chimera stage, but it has not yet been practiced, to my knowledge. Finally, the best suggestion, but also the most difficult, time-consuming, and costly, is to place the gene deletion on several different background strains (including the embryonic stem cell source), so that information regarding background modifiers can be ascertained directly.

ANTISENSE OLIGODEOXYNUCLEOTIDES

A final method for altering the expression of a gene should be considered. Based on knowledge of the target gene's sequence, it is possible to construct a short oligodeoxynucleotide (ODN) sequence that can be injected into the brain. These ODNs can hybridize to the complementary DNA (cDNA) of the gene, preventing protein synthesis. Such treatments are akin to a temporary knockout and have the obvious advantage of being useful in adult animals that have not experienced developmental compensations. They have been used successfully, but for reasons that are not well understood, they often fail. The treatments appear also to have a high risk of toxicity. However, for applications where pharmacological ligands are fairly nonspecific, the selectivity of an ODN may prove to be quite useful (Wehner and Bowers 1995).

FINDING NOVEL GENES RELEVANT FOR BEHAVIOR

Application of most of the methods discussed so far begins with the nomination of a candidate gene for further analysis. Two new sets of methods now permit exploration of genes not previously known to play a role. Both are being avidly pursued for multiple behaviors.

GENETIC MAPPING STRATEGIES

Behavioral responses are not controlled by a single gene. Rather, they show *multigenic* influence, and because each such gene typically only affects a relatively small part of trait variability, the genes underlying behavioral traits are often called *polygenes.* The scores of a distribution of individuals on a behavior affected by many polygenes are generally quantitatively distributed, rather than all-or-none. Because these polygenes are unknown, gene mapping is able to identify each as a region, or *locus*, on a chromosome. Hence, genetic jargon for these multiple genes of relatively small individual effect has come to be *quantitative trait loci (QTLs)*. Much effort has been devoted to identifying the "addresses" in the genome of QTLs affecting behavior. One area of active interest has been the identification of QTLs affecting drug responses; 23 have now been definitively mapped in mice, and one in rats (Crabbe et al. 1999).

Knowledge of the chromosomal regions that are known to harbor genes of importance is the first step toward positionally cloning them and elucidating their function. Specific genetic populations, and specialized animal models such as congenic strains, are being used to map their locations to finer resolution. The relative merits of some of these strategies have been reviewed elsewhere (Darvasi 1998). One of the exciting features of QTL mapping is that there is a high likelihood of finding the relevant human genes for traits studied in mice. It has been estimated that as much as 80 percent of the mouse genome leads us directly to specific locations on the human genome, due to syntenic conservation of groups of linked genes and markers during speciation.

GENE EXPRESSION ASSAYS FOR ASCERTAINING NOVEL GENES

Many different strategies have been developed during the last few years for identifying genes whose expression is changed by a treatment or a drug. The basic idea is to compare cDNA or RNA, representing genes actively expressed at the time, from animals exposed to drug versus those not exposed, to identify differentially expressed genes. The original method to accomplish this was termed "subtractive hybridization," which captures the flavor of the endeavor. Other, related methods now include messenger RNA (mRNA) differential display, electronically based subtractions such as Serial Amplification of Gene Expression (SAGE), and sequencing a series of expressed sequence tags, such as those being constructed as part of the Human Genome Project. The relative merits of these different procedures largely reflect technical aspects of their workings, and a clear comparative discussion of many of their strengths and weaknesses has been published (Wan et al. 1996).

Gene expression screening has been used successfully to identify a novel neuropeptide whose expression is increased by chronic psychostimulant administration (Douglass et al. 1995), and in multiple contexts to identify genes regulated by alcohol treatment (Miles et al. 1994; Chen et al. 1997; Schafer et al. 1998). A large increment in the power of these techniques will follow shortly when the use of DNA chip technology becomes available for mouse. This method will allow virtually the entire genome of an individual mouse to be displayed on a single chip, greatly increasing the number of expression-based assays possible.

CONCLUSIONS

As the invasion of neuroscience by genetics continues apace, the variety of available techniques for manipulating the genome will continue to grow. The power of such manipulations is already evident, but each approach has intrinsic strengths and limitations. The aspiring genetic engineer will be well advised to interpret results from such experimentally altered preparations with due caution. It should not be forgotten that natural selection is busily at work, even in laboratory animal populations. In fact, it may be that those animals challenged through one or the other form of genetic manipulation (e.g., gene knockouts) are under unusually high levels of natural selective pressure directed toward maintaining their fitness. The results of such essentially evolutionary forces can be remarkable, and not directly related to usual effects of the gene on the behavior.

One major benefit of the molecular revolution is the increasing recognition of the need for cooperation among scientists with different levels of expertise. To understand the various ways that a gene might affect a behavior, one needs at least four sorts of expertise in order to make maximal use of the available tools. The molecular biological manipulation performed must be understood at a level beyond cookbook application. Understanding the more quantitative and statistical aspects of gene frequency change and gene action (including such disciplines as quantitative or population genetics) is beneficial in interpreting the gene versus background effects of gene-targeted organisms. The neuroscience discipline at which the genetic manipulation is targeted (e.g., neuropharmacology for a neurotransmitter receptor knockout) must itself be comprehended in its full complexity in order to use the techniques to best advantage. Finally, the behaviors themselves must be understood in the ethological sense. That is, they must appropriately model the underlying disease, or human trait, they are hoped to represent. Because no scientist is likely to possess all four sorts of expertise, the rise of genetics is leading to a level of collaboration linking molecular, genetic, and both cellular and behavioral scientists in exciting joint endeavors.

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Chapter 10

Understanding Adaptive Central Nervous System Responses to Ethanol by Use of Transcriptional Profiling

Michael F. Miles, M.D., Ph.D.

KEY WORDS: central nervous system; ethanol; chronic AODE (effects of AOD [alcohol or other drug] use, abuse, and dependence); gene transcription; gene expression; AOD tolerance; molecular genetics; gene regulation; AOD sensitivity; neurobehavioral theory

Drug addiction likely results from the accumulation of progressive neuroadaptive events that occur in the central nervous system (CNS) in response to chronic use of abused drugs. All abused drugs activate the mesolimbocortical dopaminergic pathway, an event thought to underlie the rewarding properties of these drugs. However, chronic use generates new behavioral responses in animals and humans. These new behavioral responses include tolerance, dependence, and sensitization. These responses represent forms of CNS plasticity, much akin to "memory" resulting from other CNS responses to environmental stimuli. Importantly, tolerance/dependence and sensitization appear to alter the rewarding properties of abused drugs. Koob and others have proposed that this results in a spiral of increasing hedonic properties of abused drugs, which eventually produces another new behavior, drug addiction (Koob and Le Moal 1997).

Adaptive changes in gene expression could change neuronal functioning in specific brain regions; thus generating altered behavioral responses. Changes in gene expression refer to an alteration in the amount of protein product that is coded for by a certain gene. In eukaryotes, gene expression

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is most often regulated at the level of messenger RNA (mRNA) abundance. Thus, changing the amount of mRNA synthesized from a given gene will alter the abundance of the protein, with consequent functional changes in the cell. Prior results from our laboratory and others document that chronic ethanol exposure causes changes in neuronal gene expression in cultured cells or rodent brain (Mochly-Rosen et al. 1988; Buck et al. 1991; Gayer et al. 1991; Miles et al. 1991; Montpied et al. 1991; Mhatre and Ticku 1992; Miles et al. 1992; Charness et al. 1993; Miles et al. 1993, 1994; Devaud et al. 1997). However, specific functional consequences resulting from these changes in gene expression have not been rigorously proven.

Perhaps the best candidate for gene expression changes producing behavioral consequences occurs with the ligand-gated ion channels responding to glutamate or gamma-aminobutyric acid (GABA). Acute ethanol exposure is known to alter the function of these receptors (Suzdak et al. 1986; Lovinger et al. 1990; Ticku 1990; Wafford et al. 1990; Mihic et al. 1997), and these actions are thought to contribute to the acute intoxicating effects of ethanol. With chronic ethanol exposure, behavioral tolerance and dependence develop. A number of studies have identified changes in expression of GABA or glutamate receptor subunits accompanying chronic ethanol exposure (Buck et al. 1991; Montpied et al. 1991; Mhatre and Ticku 1992; Trevisan et al. 1994; Fitzgerald et al. 1996). It has been suggested that these changes in receptor subunit gene expression contribute to development of tolerance and dependence.

Tolerance and dependence are behaviors that develop and decay over the course of several days in rodents or humans. Changes in gene expression alone could perhaps account for these behaviors. However, sensitization and addiction are very long-lasting, which suggests that structural changes might be required for the generation of synaptic plasticity underlying these behaviors. Again, the parallel with forms of plasticity occurring with memory and learning is appropriate. Long-term memory has been proposed to require initial changes in gene expression which consequently generate structural alterations in neurons that result in a very long-lasting modification of brain function (Bolshakov et al. 1997).

Reliance upon “candidate gene” studies, such as with GABA or glutamate receptor subunits, is unlikely to produce a thorough understanding of the pleiotropic actions of ethanol on CNS function. Our laboratory has therefore chosen experimental approaches that allow a “nonbiased” identification of gene expression changes occurring in neural cells with ethanol exposure. When coupled with pharmacological or genetic approaches to correlate gene expression changes with behavior, large-scale efforts at nonbiased identification of ethanol-responsive genes (EtRGs) may complement molecular genetic studies for identifying genes underlying ethanol-related behaviors (figure 1). This chapter describes some of our previous work in this area and introduces current studies using a new technology, high-density oligonucleotide arrays. Such DNA arrays allow, for the first time, simultaneous study of ethanol action on expression of thousands of genes. This offers the possibility of understanding, at a previously unapproachable level, the coordinate effects of ethanol on neuronal function.

Our laboratory currently uses three model systems for study of drug abuse–related gene expression: (1) human or rodent neuroblastoma cell cultures exposed to ethanol (a model for gene expression changes leading to tolerance/dependence), (2) mice exposed to chronic ethanol vapor or injection (tolerance/dependence), and (3) mice exposed to repeated intraperitoneal injections of cocaine or ethanol so as to produce locomotor sensitization. Our long-term goals include understanding the mechanism of ethanol-regulated gene expression and how identified genes function to produce behaviors seen with drug abuse. Such work might eventually produce new therapeutic or diagnostic targets for treatment of alcohol abuse and alcoholism.

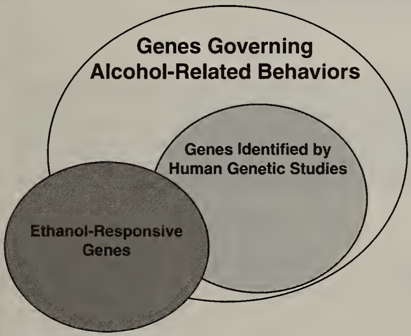
Genes Governing Alcohol-Related Behaviors
Genes Identified by Human Genetic Studies
Ethanol-Responsive Genes

Figure 1. Venn diagram indicating possible relationship of gene expression studies with genetic efforts at identifying genes underlying ethanol-related behaviors. It is proposed that only a subset of genes showing changes in expression with ethanol will actually play a role in ethanol-related behaviors. By definition, genetic studies on alcoholism (excluding studies on end-organ toxicity such as cirrhosis) will identify genes related to behaviors. A subset of ethanol-responsive genes relevant to behavior may not be accessible by genetic studies in humans.

STUDIES IN NEUROBLASTOMA CELL CULTURES

Previously, we used subtractive cloning and candidate gene approaches to study ethanol regulation of gene expression in NG108-15 neuroblastoma cell cultures (figure 2) (Miles et al. 1991, 1992, 1993, 1994). This work showed for the first time that ethanol could selectively alter gene expression at the level of gene transcription. Furthermore, we identified a group of related EtRGs (Hsc70, GRP78, and GRP94) known as molecular chaperones. The protein products of these genes are crucial for the correct processing and maturation of other proteins within the cell. We suggested that the coordinate regulation of several molecular chaperones by chronic ethanol indicates that protein trafficking is an important site of ethanol action in neural cells (Miles et al. 1994). The nonbiased approach of subtractive cloning allowed the identification of this “pattern” of EtRGs. This work also allowed the study of the promoter elements governing ethanol induction of these molecular chaperones (Miles et al. 1991; Hsieh et al. 1996).

Although our subtractive hybridization studies identified a considerable number of genes that respond to ethanol, it was still difficult to generate an appreciation of how ethanol might alter the overall function of a neuron. This was due to the fact that, once "discovered" by subtractive cloning, we still had to study each gene one at a time through such methods as Northern or Western blotting. Furthermore, subtractive hybridization studies frequently only detect genes that are expressed at moderate to high levels and show very large changes in abundance. We have, therefore, recently turned to the use of high-density oligonucleotide arrays (Affymetrix, Santa Clara, CA) for the study of gene regulatory events occurring with drug abuse (figure 3). These arrays allow the simultaneous study of the expression of thousands or tens of thousands of genes.

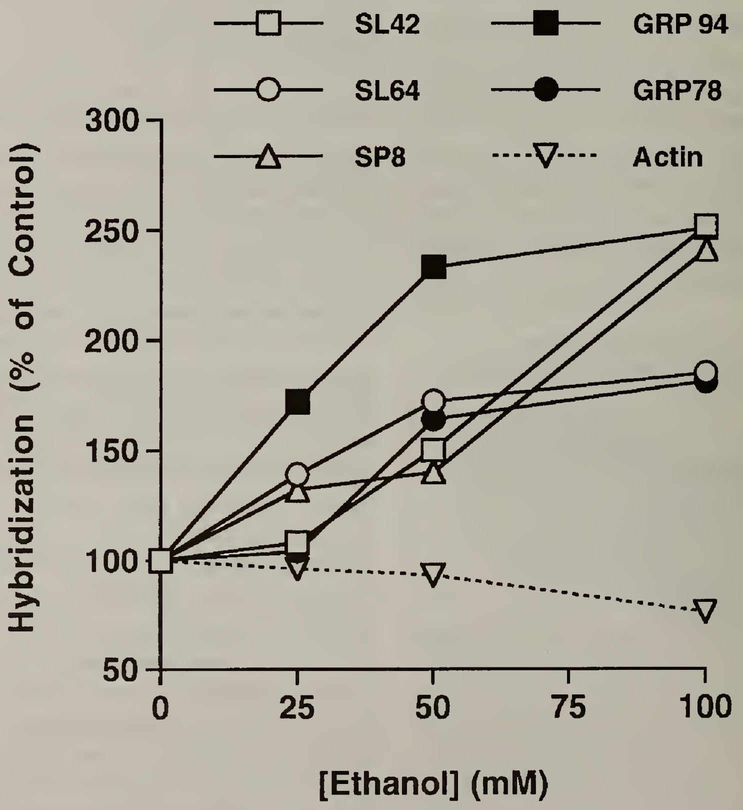

Figure 2. Ethanol-responsive genes identified by subtractive hybridization studies in NG108-15 cells; results of Northern blot studies with RNA isolated from NG108-15 cells exposed to the indicated concentrations of ethanol for 48 hours. RNA was hybridized to probes for various ethanol-responsive genes identified by subtractive hybridization (Miles et al. 1994). Actin is included as a negative control. Following hybridization and autoradiography, results were quantified by densitometry. Results are representative of experiments repeated three times.

Essentially, these "chips" have DNA oligonucleotides corresponding to particular genes synthesized on a solid surface so as to generate a microarray. In the space of approximately 1 cm² area, DNA corresponding to thousands of different genes can be synthesized at discrete, addressable locations. Biotin-labeled mRNA molecules from control (untreated) or ethanol-treated cells are then hybridized to the array. The amount of mRNA for a particular gene is determined simply by scanning the array with a specialized microscope to determine the amount of fluorescence occurring over each gene

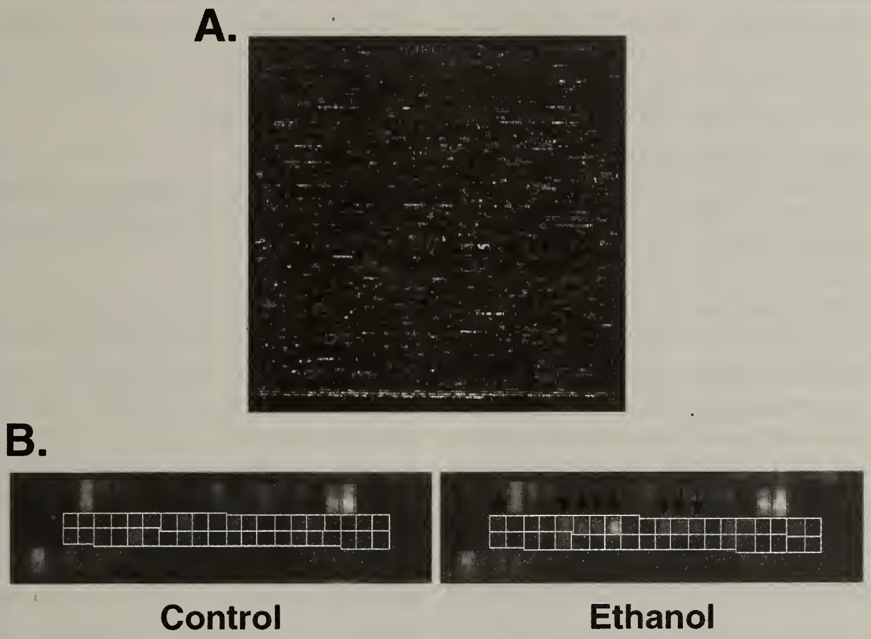

Figure 3. Analysis of ethanol-responsive gene expression in SH-SY5Y human neuroblastoma cells by high-density oligonucleotide arrays. RNA was isolated from SH-SY5Y cells treated with or without ethanol (100 mM) for 72 hours. Biotinylated complementary RNA probes were then prepared and hybridized to high-density oligonucleotide arrays according to Affymetrix protocols (Lockhart et al. 1996). Biotinylated RNA hybridizing to the immobilized oligonucleotide probes was then detected by reaction with streptavidin-phycoerythrin (Molecular Probes) followed by analysis with a dedicated scanning confocal microscope. (A) Image of fluorescent RNA hybridizing to a single chip containing probes for approximately 1,680 human genes. (B) Enlarged sections of two chips hybridized with probes prepared from control or ethanol-treated SH-SY5Y cells. The boxed features are the 40 different oligonucleotide probes for a single gene. Each gene has 20 different specific oligonucleotide probes (upper row of boxes) and 20 control oligonucleotides (lower row of boxes) that differ only by a single base-pair from the specific probes (Lockhart et al. 1996). Arrows point to specific oligonucleotides that show strong increases in hybridization intensity with RNA from ethanol-treated cells. Oligonucleotides not boxed correspond to probes for other genes showing no response to ethanol in this region of the chip.

on the chip. By comparing the results for each gene between the control and ethanol-treated samples, one obtains a virtually instantaneous "transcriptional profile" of how the cells are responding to ethanol.

Gene arrays were first used to study ethanol-responsive gene expression in a neuroblastoma cell culture model (see part B of figure 3). Following treatment of SH-SY5Y human neuroblastoma cells with 100 mM ethanol for 72 hours, array studies showed that 21 out of approximately 6,800 genes studied had a greater than twofold increase or decrease in mRNA abundance (Thibault et al. unpublished manuscript). The majority of these EtRGs function in signal transduction, but genes functioning in gene expression and cellular metabolism were also represented (data not shown). Importantly, no genes known to function in regulation of the cell cycle were regulated by chronic ethanol in these cell cultures.

Efforts to gather "functional" information about EtRGs identified on the array studies have included statistical analysis by hierarchical clustering. This procedure seeks to identify relationships between groups (genes) based on the similarity of their responses to varying stimuli. In our case, this entailed different experiments studying ethanol concentration response, time course, or alcohol chain length. When all of this DNA array data was subjected to cluster analysis, nine potential groups of genes were identified. Importantly, one group contained several genes having obviously related functions in neurotransmitter synthesis and release. Most recently, we have identified an ethanol-responsive portion of the promoter for one of these genes. A protein has been identified that binds to this region of the promoter and shows dramatic increases in DNA binding following ethanol treatment. Further characterization of this protein may allow an understanding of how ethanol regulates the expression of this and other genes important for behavioral responses to ethanol.

STUDIES ON SENSITIZATION TO COCAINE AND ETHANOL IN MICE

Recently, much of our attention has shifted to the study of cocaine or ethanol sensitization in mice. Sensitization refers to an increased response to drugs of abuse following repeated administration (Kalivas and Stewart 1991). Sensitization is frequently studied by measuring locomotor activity following drug administration. At appropriate doses, all drugs of abuse will cause an increase in locomotor activity. The gradual increase in this response with repeated drug exposure is termed "sensitization" or "reverse tolerance." Strikingly, sensitization will persist for weeks to years following drug removal. It thus represents a long-term form of CNS plasticity formed consequent to repeated drug exposure. Other studies show that sensitized animals appear to have an increased reward response to drugs of abuse. Thus, sensitization may represent an important adaptive response that predisposes an animal to abusive drug administration.

In collaboration with Dr. Tamara Phillips (Oregon Health Sciences University) and Dr. Clyde Hodge (University of California, San Francisco), we have initiated studies on ethanol and cocaine administration using the oligonucleotide array technology described above. Multiple brain regions were microdissected from animals treated with saline, acute drug, or chronic drug. All animals were studied behaviorally to document the formation of sensitization. RNA isolated from harvested brain regions was analyzed by oligonucleotide microarrays containing approximately 6,500 mouse genes (Affymetrix, Santa Clara, CA).

Our initial studies with cocaine sensitization have identified genes that are only regulated in the sensitized animals. Thus, these studies have already identified a unique set of genes that do not show changes with acute drug administration but do so following sensitization. Although a number of brain regions show changes in various signaling molecules, the prefrontal cortex show striking changes in the expression of genes with possible functions in synaptic remodeling. This is exactly the type of response we predicted. Since sensitization is a very long-lasting behavior, it should entail structural changes in the organization of neurons in certain brain regions. Our DNA array studies document evidence for such structural reorganization. Further analysis of the cellular origin of the genes showing changes may document particular neural pathways involved in sensitization. Furthermore, in ongoing work, we seek to rigorously correlate changes in gene expression with the behavior of sensitization by carrying out studies in various lines of animals or animals treated with various pharmacological agents so as to modify the degree of sensitization. Such studies will allow a detailed molecular understanding of this form of CNS plasticity that may have an important role in the formation of addictive behavior.

CONCLUSION

Our studies on regulation of gene expression have already provided new insights into the molecular basis for various behaviors induced by ethanol or other drugs of abuse. DNA array technology coupled with rigorous behavioral, genetic, and pharmacological studies will allow an unparalleled understanding of the molecular basis for the complex neuroadaptive responses that constitute drug addiction.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institute on Alcohol Abuse and Alcoholism and by intramural funding from the Ernest Gallo Clinic and Research Center. In addition, oligonucleotide array studies were initiated as a collaborative project together with Drs. Chaoqiang Lai and David Lockhart at Affymetrix (Santa Clara, CA). Norbert Wilke and Dr. Lai of Affymetrix conducted all of the array work described here. Dr. Christelle Thibault performed data analysis for array studies on SH-SY5Y cells. Dr. Elaine Shen did behavioral studies on cocaine sensitization with the assis-

tance of Dr. Tamara Phillips (Oregon Health Sciences University).

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Chapter 11

Muscarinic Control of the Immediate-Early Gene Expression

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Ulrik Fried, M.S., and Christer Larsson, Ph.D.

KEY WORDS: muscarinic receptor; gene expression; cell signaling; AODE (effects of AOD [alcohol or other drug] use, abuse, and dependence); carbachol; mRNA; central nervous system; AOD sensitivity; laboratory study

Acetylcholine in the brain is generally thought to be an excitatory neurotransmitter, which acts on nicotinic or muscarinic receptors. The nicotinic receptor is a ligand-gated ion channel, whereas the muscarinic receptor belongs to the G protein–coupled receptor superfamily. Nicotinic receptors are sparser in the brain than muscarinic receptors, and most of the behavioral effects associated with cholinergic pathways seem to be related to acetylcholine acting on muscarinic receptors. It is well known that the muscarinic receptors in the brain are important for the process of learning and memory (Bartus et al. 1982; Coyle et al. 1983; Hagan and Morris 1988).

Effects of ethanol on the level of acetylcholine and the function of muscarinic receptors in the central nervous system have been extensively studied (Nordberg and Wahlström 1992). Acute ethanol inhibited muscarinic receptor function in primary cortical cultures (Kovacs et al. 1995), in a neuronal cell line (Larsson et al. 1995), and in rat brain (Candura et al. 1992; Balduini et al. 1994; Tan and Costa 1995). On the other hand, chronic exposure to ethanol up-regulated muscarinic receptor function and increased

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the muscarinic receptor binding sites in neuronal cells (Kim et al. 1993; Larsson et al. 1996). Hu and colleagues (1993) reported that chronic ethanol up-regulated expression of muscarinic receptor genes. Thus, the muscarinic signaling seems to be involved in the process of ethanol-induced changes in neuronal cells.

The *fos* and *jun* gene family consists of immediate-early genes encoding transcription factors, which dimerize with each other forming the complex of activator protein 1 (AP-1). AP-1 regulates expression of a number of genes in the nervous system by acting on the AP-1 *cis* element flanking these genes (Morgan and Curran 1991; Hughes and Dragunow 1995). Acetylcholine receptor–stimulated expression of the *fos* and *jun* genes has been demonstrated both in cell lines and in animal models (Greenberg et al. 1986; Blackshear et al. 1987; Trejo and Brown. 1991; Weiner et al. 1991; Chou et al. 1992; Trejo et al. 1992; Fukamauchi et al. 1993; Hughes and Dragunow 1993). Specific patterns of immediate-early gene expression in rat brain were observed after stimulation of pirenzepine-sensitive muscarinic receptors (Hughes and Dragunow 1994). We demonstrated that muscarinic receptor–stimulated expression of c-*fos* was primarily mediated via M1 receptors and through protein kinase C (PKC) in SH-SY5Y cells (Larsson et al. 1994). Since the muscarinic receptor–coupled expression of *fos* and *jun* genes may have potential in regulating the transcription of other neuronal genes, thereby accounting to a certain extent for the ethanol effects on brain function, it is of interest to understand how ethanol affects the expression of these genes induced by stimulation of muscarinic receptors in neuronal cells. This chapter reports the effects of ethanol on the muscarinic receptor–stimulated expression of *fos* and *jun* genes using a human neuroblastoma cell line, SH-SY5Y.

EXPERIMENTAL PROCEDURES

Cell Culture and Ethanol Exposure

SH-SY5Y cells were cultivated in Eagle's minimal essential medium with L-glutamine and Earle's salts supplemented with 10 percent fetal calf serum, 100 IU/mL penicillin, and 100 µg/mL streptomycin in a humidified atmosphere with 5 percent CO_2 at 37 °C. Cells were allowed to grow for 1 week, reaching confluence before carrying out any treatment protocol.

For ethanol exposure, a routine protocol was used (Rodriguez et al. 1992). In brief, at least five and as many as ten 60-mm dishes containing ethanol were kept in one plastic box during ethanol exposure to prevent ethanol evaporation. To equilibrate ethanol concentration, a small dish containing 4 mL 4 percent ethanol was included in each ethanol-exposed box. Separate but identical incubators were used for ethanol-exposed dishes and for control dishes, to ensure that control cells were not influenced by evaporated ethanol from ethanol-containing dishes. Ethanol concentrations were examined before cells were harvested.

NORTHERN BLOT ANALYSIS

Gene expression was mainly analyzed with Northern blot technique. The method described by Chomczynski and Sacchi (1987) was used to isolate total cellular RNA. The RNA was then separated on agarose gel and blotted to nylon membrane with the capillary blot technique (Larsson et al. 1994). The images of ethidium bromide–stained total RNA on the gel were saved to analyze the intensity and the amount of RNA that was loaded. Membranes were prehybridized for 1 hour and then hybridized with [^{32}P]dCTP (deoxycytidine triphosphate)–labeled complementary DNA (cDNA) probes for overnight. The membranes were washed 3×10 minutes in 2, 1, and 0.1x standard saline citrate, respectively, wrapped in plastic film, and exposed to x-ray film for 2–3 days at –70 °C. Intensities in bands were quantified with the Gel Documentation System 5000 and the Soft Ware 5000 for Windows (Ultra-Violet Products, Cambridge, England), and the intensities in the messenger RNA (mRNA) band were related to the intensities in the 28S ribosomal RNA (rRNA) band from the gel.

GEL MOBILITY SHIFT ASSAY

Gel mobility shift assay was used in this study to detect the binding activity of transcription factors to DNA fragments. Cell incubation was terminated by removing the medium, and cells were lysed (10 mM Tris [pH 7.4]; 3 mM $MgCl_2$, 10 mM NaCl, 0.5 percent Tergitol NP40) and pelleted at 4,000×g for 5 minutes at 4 °C. The pellet was extracted for 30 minutes in buffer containing 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulphonic acid (HEPES), pH 7.9; 20 percent glycerol; 0.3 M NaCl; 1.5 mM $MgCl_2$; 0.2 mM ethylenediaminetetraacetic acid (EDTA); 1 mM dithiothreitol (DTT); 0.1 mM β–glycerophosphate; 0.05 mM vanadate; 1 mM phenylmethyl sulfonyl fluoride; and 1 µg/mL each of pepstatin A, leupeptin, and aprotinin. The samples were centrifuged at 15,000×g for 15 minutes at 4 °C. The supernatant containing nuclear proteins was collected and protein content was analyzed using the method described by Bradford (1976).

Double-stranded oligonucleotides were labeled with [γ-^{32}P]adenosine triphosphate. For binding reaction, the nuclear protein extract (5 µg in 5 µL) was incubated in a total volume of 10 µL in binding buffer containing 1 µg poly(dI-dC)·poly(dI-dC), 4 percent glycerol, 50 mM NaCl, 10 mM Tris (pH 7.6), 1 mM EDTA, 0.5 mM DTT, and 1 mM $MgCl_2$. After incubation for 10 minutes at room temperature, 50,000–60,000 cpm of [^{32}P]labeled oligonucleotides was added and the incubation continued for another 20 minutes at room temperature. DNA-protein complexes were resolved on a Tris-borate-EDTA–buffered 6 percent polyacrylamide gel (acrylamide:bisacrylamide, 80:1). After separation, the gel was wrapped in plastic wrap and exposed to x-ray film for 4–8 hours at –70 °C.

For gel supershift assay, 1 µg of the antibody was added in each binding reaction and the incubation was carried out at 4 °C for 1 hour to overnight. Other procedures were the same as described above.

RESULTS AND DISCUSSION

Muscarinic Receptor– and PKC-Stimulated Expression of *fos* and *jun* Genes

No mRNA level of c-*fos*, *fos*B, and *jun*B was detected in unstimulated SH-SY5Y cells, whereas the expression of c-*jun* and *jun*D was evident (Ding et al. 1996). This finding is in line with previous studies in which mRNA level of c-*jun* or *jun*D was observed in several other cell lines (Chiu et al. 1989; Hirai et al. 1989; de Groot et al. 1990; Trejo and Brown 1991; Trejo et al. 1992; Andersson et al. 1994; Haby et al. 1994).

The AP-1 binding activity was also detectable in SH-SY5Y cells, which is consistent with a previous finding in this cell line (Li et al. 1996). Two bands of AP-1 binding were found by means of gel mobility shift assay. c-Jun and JunD are very likely participating in the formation of AP-1 dimer in the unstimulated cells because they are both expressed under these conditions. In fact, inclusion of JunD antibody attenuates the AP-1 binding activity in both unstimulated and phorbol ester–treated cells, indicating that JunD is a part of the AP-1 dimer in both cases.

Activation of muscarinic receptors elicits expression of c-*fos* in SH-SY5Y cells (Larsson et al. 1994). In 1321-N1 astrocytoma cells, the expression of c-*fos*, c-*jun*, and *jun*B induced by carbachol

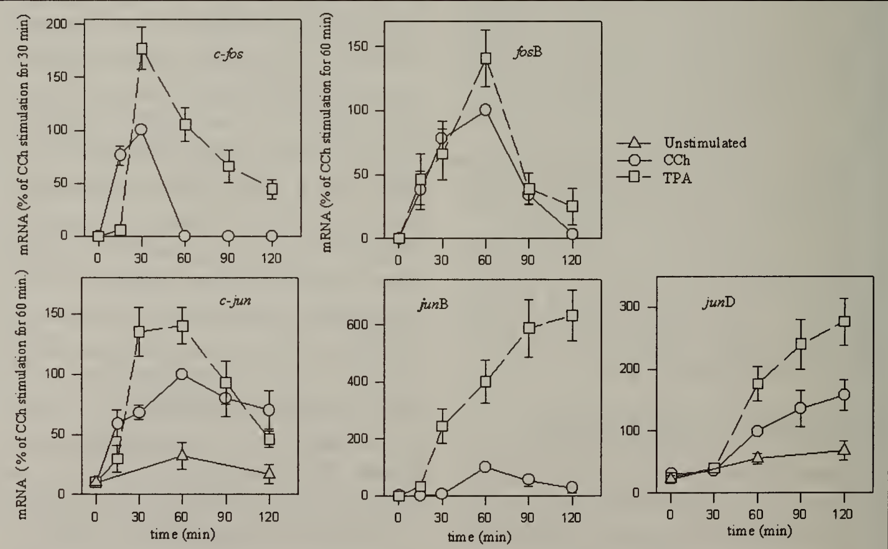

Figure 1. Time course of carbachol(CCh)- and 12-*O*-tetradecanoylphorbol-13-acetate (TPA)–stimulated increase in c-*fos*, *fos*B, c-*jun*, *jun*B, and *jun*D messenger RNA (mRNA) levels. SH-SY5Y cells were stimulated with 1 mM CCh or 1 µM TPA for different time intervals. Reprinted with permission from Ding, W.Q.; Larsson, C.; and Alling, C. Stimulation of muscarinic receptors induces expression of individual *fos* and *jun* genes through different transduction pathways. *Journal of Neurochemistry* 70(4):1722–1729, 1998.

stimulation has also been demonstrated (Blackshear et al. 1987, Trejo and Brown. 1991). We found that expression of c-*fos*, *fos*B, c-*jun*, *jun*B, and *jun*D was induced by carbachol stimulation in SH-SY5Y cells (figure 1) (Ding et al. 1998*c*). The expression of individual genes displayed different temporal patterns, indicating that these inducible genes are differentially regulated upon activation of muscarinic receptors.

Stimulation with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) also induced expression of c-*fos*, *fos*B, c-*jun*, and *jun*D with temporal patterns that were similar to the carbachol-induction. However, the TPA-stimulated increase in *jun*B mRNA level was substantially larger and longer lasting compared with carbachol stimulation (Ding et al. 1998*c*). The mechanism behind this phenomenon is unclear, but it may be explained by the fact that the regulation of *jun*B expression differs from the regulation of other *fos* and *jun* genes (de Groot et al. 1991; Mellström et al. 1991; Carter 1993; Menegazzi et al. 1994; Pelto-Huikko et al. 1995). The temporal expression pattern of *jun*B in the brain has also been demonstrated to be different from that of c-*jun* and *jun*D (Herdegen et al. 1995).

Stimulation of muscarinic receptors with carbachol and activation of PKC with TPA both dramatically increased the AP-1 binding activity (figure 2) (Ding et

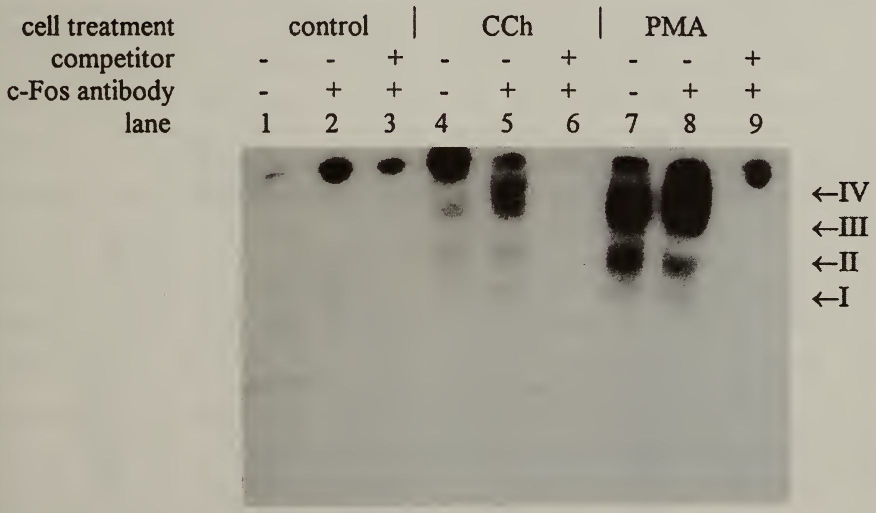

Figure 2. Fos/AP-1 binding activity in SH-SY5Y cells that had been stimulated with 1 mM carbachol (CCh) or 1 μM phorbol ester revealed by gel mobility supershift assay. Bands I and II were detected in unstimulated cells. After stimulation a new complex bound to the AP-1 oligonucleotide (band III) was seen, and upon inclusion of c-Fos antibody a fourth complex (band IV) was detected. Reprinted from *Brain Research. Molecular Brain Research*, Vol. 46, Ding, W.Q.; Larsson, C.; Simonsson, P.; and Alling, C., Effects of ethanol on muscarinic receptor-stimulated *c-fos* expression in human neuroblastoma cells, pp. 77–84. Reproduced with permission from Elsevier Science, copyright 1997.

al. 1997). New transcription factors were synthesized upon carbachol and TPA stimulation, which was manifested as a new AP-1 binding band detected by gel mobility shift assay. At least three of the Fos and Jun proteins, c-Fos, FosB, and JunD, were involved in the formation of AP-1 dimer under this condition, as measured by the supershift assay (Ding et al. 1997). The time course of carbachol-stimulated increase in AP-1 binding activity in SH-SY5Y cells was the same as previously reported (Li et al. 1996).

Theoretically, carbachol stimulation will activate both muscarinic and nicotinic receptors, resulting in gene expression (Greenberg et al. 1986). The expression of individual *fos* and *jun* genes in response to carbachol stimulation observed in this study is due to the involvement of muscarinic receptors, because pretreatment with atropine completely blocked the carbachol-induced gene expression (Ding et al. 1998*c*). We have previously demonstrated that the carbachol-stimulated increase in the formation of inositol 1,4,5-trisphosphate (IP$_3$), diacylglycerol and in the expression of c-*fos* were mediated via M1 receptors (Larsson et al. 1994, 1995). Thus, the *fos* and *jun* genes investigated in this study seem to be primarily mediated by M1 receptors after stimulation with carbachol.

INTRACELLULAR SIGNALING PATHWAYS INVOLVED IN THE MUSCARINIC RECEPTOR–STIMULATED EXPRESSION OF *FOS* AND *JUN* GENES

The intracellular pathways coupling muscarinic receptor stimulation to expression of immediate-early genes are not yet identified. It has been claimed that carbachol-induced expression of c-*fos* and c-*jun* is mediated via both PKC-dependent and -independent pathways in astrocytoma cells (Blackshear et al. 1987) and that carbachol-stimulated expression of c-*fos* is primarily mediated by PKC in SH-SY5Y cells (Larsson et al. 1994). When the intracellular signaling pathways were investigated in the present study, we provided a rationale for subdividing these five genes into two groups based on the effects of kinase inhibitors on the carbachol-stimulated expression of individual *fos* and *jun* genes (Ding et al. 1998*c*). One group includes c-*fos*, *fos*B, and *jun*B, which were primarily induced via classical PKC isoforms in response to carbachol stimulation, although unknown pathways responsible for minor effects need to be further defined. Another group includes c-*jun* and *jun*D, expression of which was regulated mainly by calmodulin (CaM) kinase II upon muscarinic receptor activation.

The PKC pathway in SH-SY5Y cells has been well characterized (Larsson et al. 1992; Leli et al. 1992; Heikkila et al. 1993; Rosner et al. 1995). The existence of CaM kinase II pathway was indicated by the finding that increase in calcium level with calcium ionophore led to expression of these genes, which was inhibited by the CaM kinase II inhibitor 1-[N,O-*bis*(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62) but not by the PKC inhibitor GF109203X. Previous studies have shown that CaM kinase II is expressed in neuroblastoma SK-N-SH cells (Mangels and Gnegy 1992) and muscarinic receptor–mediated increase in intracellular calcium level is inhibited by pretreatment with KN-62 in this cell line (Puhl et al. 1997). CaM kinase II–mediated phosphorylation has also been reported to be prevented by KN-62 (Communi et al. 1997). These findings are in line with the results in our study.

Thus, at least three separate signal pathways participate in carbachol-stimulated expression of immediate-early genes: PKC-mediated pathways, CaM kinase II–mediated pathways, and unknown pathways (figure 3). The cyclic adenosine monophosphate (cAMP)–protein kinase A pathway seems to be excluded because stimulation with cAMP failed to induce expression of these genes. A possibility that mitogen-activated protein (MAP) kinase pathways are involved in these events needs to be further investigated.

The differences in the promoter structure among these genes may to a certain extent explain the separate pathways that have been used by activation of muscarinic receptors to induce expression of respective genes. For instance, c-*fos*, *fos*B, and *jun*B genes contain SRE (serum responsive element) in their promoter region, which has been shown to be responsible for the induction of these genes. The remarkable difference between the carbachol- and TPA-induced expression of *jun*B may be attributed to the complexity of the promoter structure through which multiple

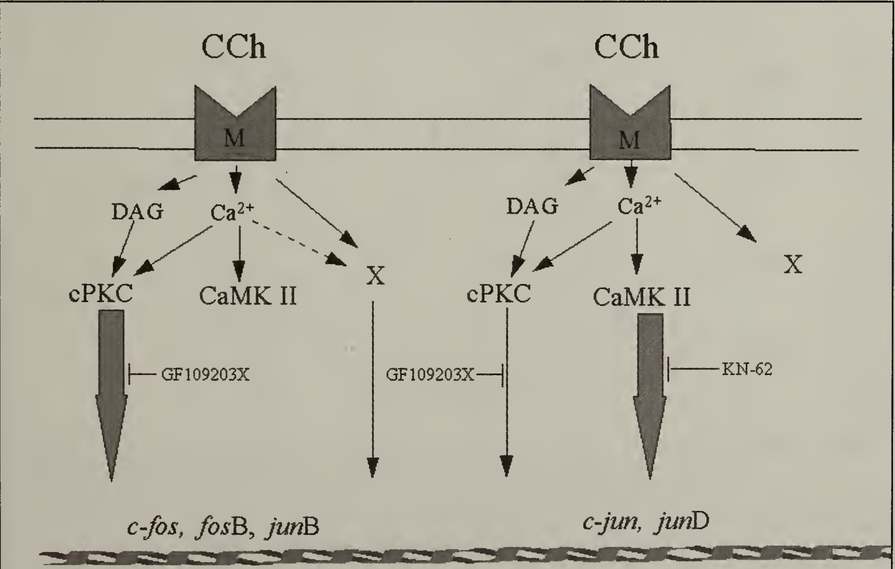

Figure 3. Proposed signaling pathways coupling muscarinic receptor stimulation to the expression of immediate-early genes in SH-SY5Y cells. CaMK II = calmodulin kinase II; CCh = carbachol; cPKC = classical protein kinase C; DAG = diacylglycerol; M = muscarinic receptor; X = unknown signaling substances.

elements were required to achieve maximal serum induction of *jun*B (Phinney et al. 1996). On the other hand, there is no SRE in c-*jun* and *jun*D promoter. Induction of c-*jun* is usually mediated through the AP-1 elements, and the inducibility of *jun*D has been less investigated and is still a matter of controversy (Angel et al. 1988; Hirai et al. 1989; Ryder et al. 1989). Since there is an AP-1 element in the *jun*D promoter (Berger and Shaul 1994), it is plausible that *jun*D shares a mechanism of induction with c-*jun* in response to carbachol stimulation in SH-SY5Y cells.

EFFECTS OF ETHANOL ON THE BASAL EXPRESSION OF *FOS* AND *JUN* GENES

The mRNA levels of c-*fos*, *fos*B, and *jun*B could not be detected in either control or ethanol-exposed SH-SY5Y cells, indicating that ethanol alone cannot initiate expression of these genes (Ding et al. 1996). On the other hand, the basal mRNA levels of c-*jun* and *jun*D were up-regulated in cells that had been exposed to ethanol for 2 and 4 days in a dose- and time-dependent manner (figure 4), suggesting that these two genes in neuronal cells are sensitive to ethanol. The up-regulation of the expression of c-*jun* and *jun*D by ethanol is most likely due to an increased transcription of the genes, because the degradation of c-*jun* and *jun*D mRNA was unaffected in experiments with actinomycin D (Ding et al. 1996). It is now well accepted that ethanol may modulate gene expression, possibly via influencing the transcription of the genes (Diamond and Gordon

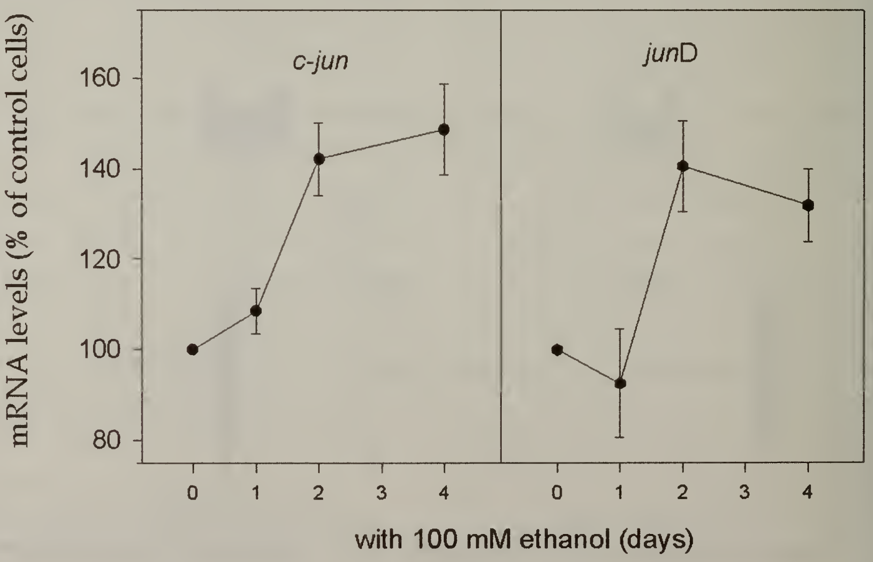

Figure 4. Effect of ethanol on expression of the c-*jun* and *jun*D genes. SH-SY5Y cells were exposed to 100 mM ethanol for 1, 2, or 4 days before messenger RNA (mRNA) levels were determined. Reprinted with permission from Ding, W.Q.; Larsson, L.; and Alling, C. Ethanol exposure increases expression of *c-jun* and *junD* in human neuroblastoma cells. *Neuroreport* 7(13):2191–2195, 1996.

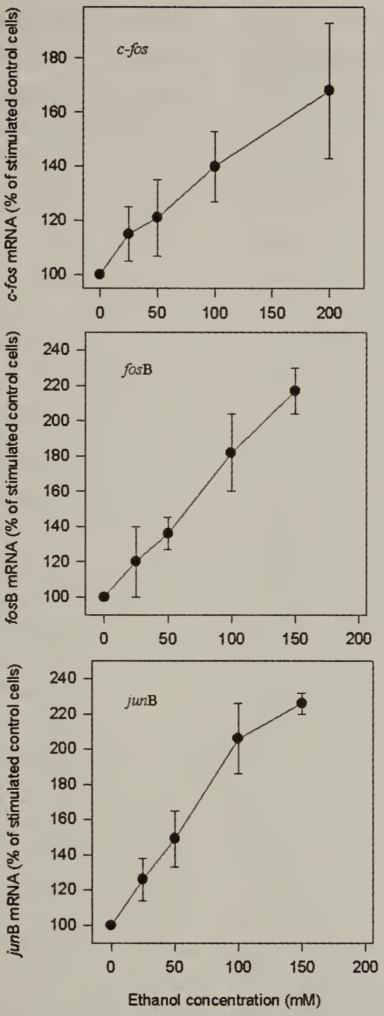

Figure 5. Effect of increasing concentration of ethanol on a carbachol-stimulated increase in c-*fos*, *fos*B, and *jun*B messenger RNA (mRNA) levels. SH-SY5Y cells were exposed to increasing concentrations of ethanol for 4 days before 1 mM carbachol stimulation for 60 minutes (100 µM for 30 minutes were used for c-*fos*). Reprinted from *Brain Research. Molecular Brain Research*, Vol. 46, Ding, W.Q.; Larsson, C.; Simonsson, P.; and Alling, C., Effects of ethanol on muscarinic receptor-stimulated *c-fos* expression in human neuroblastoma cells, pp. 77–84. Reproduced with permission from Elsevier Science, copyright 1997, and from Ding, W.Q.; Fried, U.; Larsson, C.; and Alling, C. Ethanol exposure potentiates *fos*B and *jun*B expression induced by muscarinic receptor stimulation in neuroblastoma SH-SY5Y cells. *Alcoholism: Clinical and Experimental Research* 22(1):225–230, 1998.

1997), a conclusion that favors the present findings. In contrast to long-term ethanol exposure, acute ethanol treatment selectively inhibited expression of *jun*D, whereas mRNA levels of c-*jun* were unaffected (Ding et al. 1998*a*).

EFFECTS OF ETHANOL ON MUSCARINIC RECEPTOR–STIMULATED EXPRESSION OF *FOS* AND *JUN* GENES

Exposure to ethanol was not sufficient to induce the expression of c-*fos*, *fos*B,

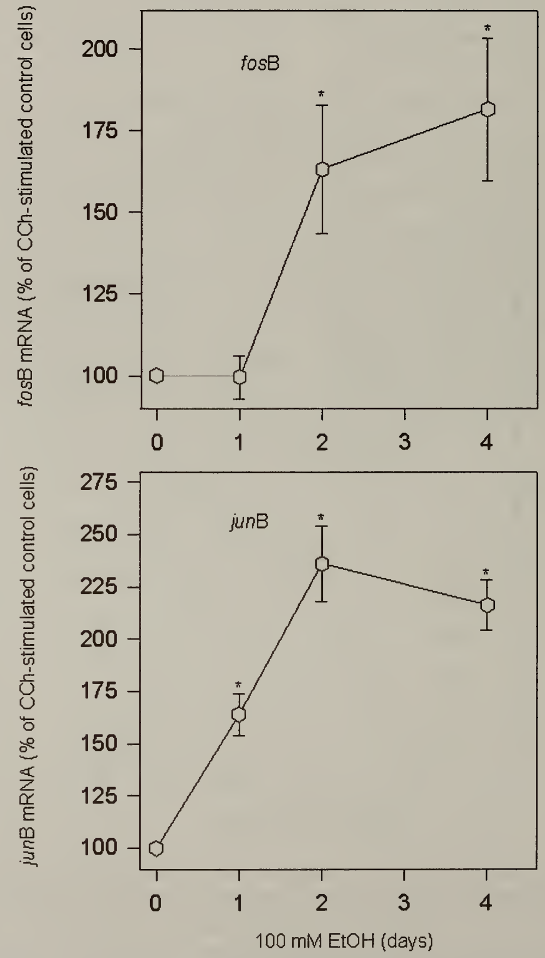

Figure 6. Influence of time dependence of ethanol (EtOH) exposure for the potentiation of carbachol (CCh)-stimulated (1 mM for 60 minutes) *fos*B and *jun*B messenger RNA levels in SH-SY5Y cells. Reprinted with permission from Ding, W.Q.; Fried, U.; Larsson, C.; and Alling, C. Ethanol exposure potentiates *fos*B and *jun*B expression induced by muscarinic receptor stimulation in neuroblastoma SH-SY5Y cells. *Alcoholism: Clinical and Experimental Research* 22(1):225–230, 1998.

and *jun*B in SH-SY5Y cells, but the carbachol-stimulated expression of these genes was up-regulated after long-term ethanol treatment (figures 5 and 6) in a dose- and time-dependent manner (Ding et al. 1997, 1998*b*). We have previously demonstrated that long-term ethanol exposure potentiates the muscarinic receptor–stimulated IP_3 formation and increases muscarinic receptor binding sites in these cells (Larsson et al. 1996). The results from the present study further indicate that ethanol potentiates the muscarinic signaling, leading to an up-regulated gene expression. Because the increase in c-Fos, FosB and JunB proteins may have profound effects on their target gene expression, thereby regulating cellular function, these findings support the notion that the muscarinic receptor system may play an important role in ethanol-induced alterations of brain function.

Among these three genes, carbachol-induced expression of *jun*B seems to be more sensitive to the length of time of ethanol exposure. Whereas 2 days of ethanol exposure were required to potentiate c-*fos* and *fos*B expression induced by carbachol stimulation, 1 day was sufficient for the up-regulation of the *jun*B mRNA level (Ding et al. 1998*b*). The mechanisms for the temporal difference of these three genes upon ethanol exposure are unclear. A plausible explanation would be that muscarinic receptor–mediated *jun*B expression is partially regulated via a pathway that is not used by c-*fos* and *fos*B, and that this pathway is more sensitive to ethanol. The temporal difference of the ethanol potentiation on these three genes suggests that carbachol-induced AP-1 dimer may be composed of a variety of different monomers after ethanol exposure for different time periods. This may provide more diverse effects on the AP-1 target gene regulation. The alteration of AP-1 dimer has been demonstrated in rat brain after chronic cocaine treatment (Hope et al. 1994), in which an increase in the short form of FosB in the AP-1 complex was detected, and similar phenomena may appear during ethanol exposure.

A decrease in mRNA levels of c-*fos*, *fos*B, and *jun*B induced by carbachol stimulation was detected after acute ethanol exposure (table 1). The carbachol-stimulated increase in Fos/AP-1 binding activity was also attenuated by acute ethanol (figure 7). These findings are well in line with previous reports that acute ethanol attenuated muscarinic receptor signaling in different cell systems (Hoffman et al. 1986; Smith et al. 1986; Sanna et al. 1994). We previously demonstrated that muscarinic receptor–stimulated increase in IP_3 formation is inhibited in SH-SY5Y cells acutely exposed to ethanol (Larsson et al. 1995). However, the inhibition of gene expression observed in this study may not be associated with the inhibition of IP_3 formation, because only the peak of IP_3, which was obtained 10 seconds after carbachol stimulation, was sensitive to acute ethanol, whereas 5 minutes of carbachol stimulation were required for the induction of c-*fos* in these cells (Larsson et al. 1994). The observation that phorbol ester–stimulated expression of c-*fos* was also inhibited by acute ethanol in

Table 1. Attenuation by Acute Ethanol of Muscarinic Receptor–Stimulated Expression of c-*fos*, *fos*B, and *jun*B.

	mRNA Level (% of Carbachol-Treated Cells)		
Treatment	**c-*fos***	***fos*B**	***jun*B**
Carbachol (1 mM)	100	100	100
Ethanol (100 mM)	66 ± 10*	62 ± 10*	76 ± 6*

Note: SH-SY5Y cells were exposed to 100 mM ethanol for 15 minutes prior to 1 mM carbachol stimulation for 30 minutes (c-*fos*) or 60 minutes (*fos*B and *jun*B). mRNA = messenger RNA. * $p < 0.01$.

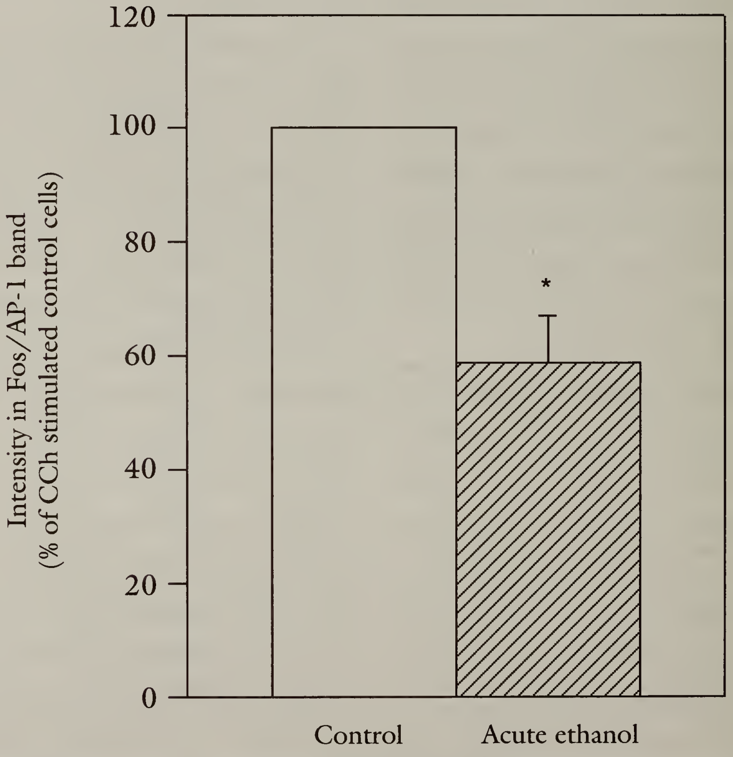

Figure 7. Effect of acute ethanol exposure on carbachol (CCh)-stimulated Fos/AP-1 binding activity. SH-SY5Y cells were stimulated with 100 μM CCh in the absence or presence of 100 mM ethanol and subjected to gel mobility supershift assay. Reprinted from *Brain Research. Molecular Brain Research*, Vol. 46, Ding, W.Q.; Larsson, C.; Simonsson, P.; and Alling, C., Effects of ethanol on muscarinic receptor-stimulated *c-fos* expression in human neuroblastoma cells, pp. 77–84. Reproduced with permission from Elsevier Science, copyright 1997.

these cells indicates that acute ethanol may influence the muscarinic signaling via a mechanism that differs from what is seen in long-term ethanol effects.

In conclusion, we have demonstrated that stimulation of muscarinic receptors induces expression of *fos* and *jun* genes through different transduction pathways in SH-SY5Y cells, and that muscarinic receptor–coupled expression of immediate-early genes is sensitive to both acute and chronic ethanol exposure. These findings may indicate one of the mechanisms by which ethanol induces neuroadaptation. We also showed that the basal and TPA-stimulated expression of *fos* and *jun* genes could be selectively altered by ethanol, suggesting different sensitivity of immediate-early genes in response to ethanol treatment in the nervous system.

ACKNOWLEDGMENTS

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Chapter 12

Protein Kinase C Delta and Epsilon in Neuronal Responses to Chronic Ethanol Exposure

Robert O. Messing, M.D.

Key words: protein kinases; chronic AODE (effects of AOD [alcohol or other drug] use, abuse, and dependence); ethanol; voltage gated channel; calcium channel; biological regulation; growth promoting factors; neuron; addiction; laboratory study

Protein kinase C (PKC) is a multigene family of phospholipid-dependent, serine-threonine kinases central to many signal transduction pathways; so far, 10 family members encoded by 9 different genes have been described (α, βI, βII, γ, δ, ε, ζ, η, ι, and θ) (Nishizuka 1992; Selbie et al. 1993). Based on biochemical properties and sequence homology, the PKC family has been divided into three groups: "conventional" cPKCs (α, βI, βII, and γ) regulated by calcium and diacylglycerols or phorbol esters; "novel" nPKCs (δ, ε, η, and θ), which are calcium independent but sensitive to diacylglycerol and phorbol myristate acetate (PMA); and "atypical" aPKCs (ζ and ι/λ), which are insensitive to calcium, diacylglycerol, and PMA.

We have found that chronic exposure to 50–200 mM ethanol for 2–8 days increases PKC activity in the neural cell lines PC12 and NG108-15 (Messing et al. 1991*b*). In PC12 cells, this is associated with increased high-affinity phorbol ester binding and PKC-mediated phosphorylation (Messing et al. 1991*b*) and with a selective increase in immunoreactivity (Messing et al. 1991*b*) and messenger RNA (mRNA) (Roivainen et al. 1994) for two PKC isozymes, PKCδ and PKCε. Similar increases in these

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two novel PKC isozymes have been documented in NG108-15 cells exposed to 200 mM ethanol for 48 hours (Coe et al. 1996). Ethanol does not increase the formation of the PKC activator diacylglycerol or activate PKC in an in vitro assay (Messing et al. 1991*b*). These findings suggest that chronic exposure to ethanol increases PKC activity by increasing expression of PKCδ and PKCε. This chapter presents a review of our recent work regarding the role of these two isozymes in neuronal responses to chronic ethanol exposure.

PKCδ IN REGULATION OF L-TYPE Ca^{2+} CHANNELS BY ETHANOL

Voltage-gated Ca^{2+} channels mediate Ca^{2+} entry into neurons and regulate neurotransmitter release, firing patterns, gene expression, and differentiation (McClesky 1994; Ghosh and Greenberg 1995). Several types of Ca^{2+} channels have been identified, with distinct electrophysiological and pharmacological properties (Zhang et al. 1993; Randall and Tsien 1995). L-type channels are activated by high voltage, inactivate slowly, and are blocked by dihydropyridine (DHP) and phenylalkylamine Ca^{2+} channel antagonists. Several manifestations of ethanol intoxication and dependence may be due to modulation of L-type channels. In nerve terminals from rat neurohypophysis and in PC12 cells, brief exposure to intoxicating concentrations (10–50 mM) of ethanol inhibits L-type channels by decreasing open channel probability (Wang et al. 1994), promoting channel inactivation (Mullikin-Kilpatrick and Treistman 1995), and interacting with G_i (Mullikin-Kilpatrick et al. 1995). Prolonged exposure of PC12 cells to ethanol produces a reversible concentration- and time-dependent increase in K^+-evoked $^{45}Ca^{2+}$ uptake and in L-type calcium currents measured in the absence of ethanol (Messing et al. 1986; Skattebol and Rabin 1987; Grant et al. 1993). This is associated with a corresponding increase in the number of binding sites for DHP Ca^{2+} channel antagonists. These findings indicate that chronic exposure to ethanol increases expression of functional L-type channels. Similar increases in DHP binding have been detected in brain membranes from ethanol-dependent rodents (Dolin et al. 1987; Brennan et al. 1990).

In chick skeletal myocytes (Navarro 1987) and *Aplysia* bag cell neurons (Strong et al. 1987), phorbol esters that activate most PKC isozymes increase the number of functional Ca^{2+} channels. We found in PC12 cells that up-regulation of L-type channels by ethanol is also mediated by PKC since it is prevented by culturing cells with the PKC inhibitors polymyxin B or sphingosine (Messing et al. 1990). More recently, we found that the more selective PKC inhibitor GF109203X (Toullec et al. 1991) also prevents ethanol-induced increases in L-type channel function (Gerstin et al. 1997).

Since chronic exposure to ethanol selectively up-regulates PKCδ and PKCε, we examined whether these isozymes are required for ethanol-induced increases in L-type channels.

To perform these studies, we used an approach based on the work of Mochly-Rosen and colleagues (Mochly-Rosen and Gordon 1998), who showed that upon activation, PKC isozymes translocate to specific intracellular sites where they bind anchoring proteins, termed "RACKs" (receptors for activated C-kinase) (Mochly-Rosen 1995). The first RACK protein that was cloned was RACK1, which interacts with the C2 domain of cPKCs (Ron et al. 1994). The sites of interaction between RACK1 and the C2 domain of PKCβ have been mapped, and short peptides derived from these domains inhibit translocation and activation of PKCβII in cardiac myocytes (Ron et al. 1995),

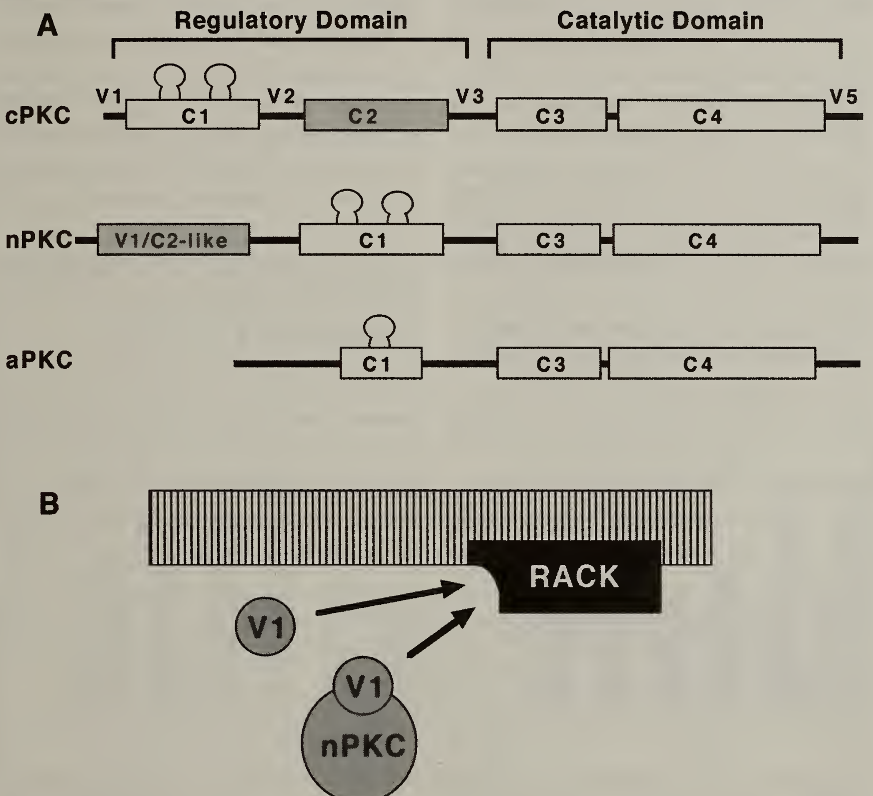

Figure 1. Novel protein kinase C (nPKC) structure and receptor for activated C-kinase (RACK) binding. (A) Schematic structure of classical PKC (cPKC), nPKC, and atypical PKC (aPKC) isozymes (adapted from Nishizuka 1992), showing variable (V) and constant (C) regions. The V1 region of nPKCs, shown in gray, has structural homology to the C2 domain of cPKCα, β, γ (also shown in gray) and hence is labeled "V1/C2-like." (B) The V1 regions of PKCδ and PKCε prevent phorbol ester–induced association of the corresponding nPKC isozyme to cell membranes and cytoskeletal elements by competing for binding to isozyme-selective RACKs. Reviewed in Mochly-Rosen and Gordon 1998.

PKCβ in *Xenopus* oocytes (Ron et al. 1995), and PKCα in pancreatic islet cells (Yedovitzky et al. 1997).

Homology has been noted between the first variable region of PKCε (εV1) and the C2 domain of cPKCs (Sossin and Schwartz 1993), suggesting that, similar to the C2 domain of cPKCs, εV1 contains a binding site for a PKCε-specific RACK (figure 1). Indeed, the cotamer protein β′COP was recently identified as a PKCε-specific RACK by expression cloning using εV1 (Csukai et al. 1997). Studies by Johnson and colleagues (1996) have shown that in cultured cardiac myocytes, εV1 and the corresponding fragment of PKCδ, δV1, specifically inhibit phorbol ester–mediated translocation of the corresponding PKC isozyme. In addition, εV1 selectively prevents phorbol ester–induced depression of contraction (Johnson et al. 1996). Thus, it appears that δV1 and εV1 compete with PKCδ and PKCε for binding to their corresponding RACKs and, as a result, inhibit PKCδ and PKCε function.

To examine whether PKCδ or PKCε is required for ethanol-induced increases in L-type channels, we created PC12 cell lines that stably express the fragments δV1 or εV1 (Gerstin et al. 1997; Hundle et al. 1997). Expression of these fragments selectively inhibited PMA-induced translocation of the corresponding PKC isozyme. We found that ethanol-induced increases in K^+ -stimulated $^{45}Ca^{2+}$ uptake and DHP binding (table 1) were markedly reduced in cells expressing the δV1 fragment but were not altered in cells expressing εV1 or the vector alone (Gerstin et al. 1997). These findings indicate that PKCδ is required for up-regulation of L-type Ca^{2+} channels by chronic exposure to ethanol.

REGULATION OF ETHANOL-INDUCED NEURAL PLASTICITY BY PKCε

Several studies with PKC-activating phorbol esters have suggested that PKC modulates neural differentiation and plasticity (reviewed in Hundle et al. 1995). There is evidence indicating that PKCε specifically plays a role in these

Table 1. Expression of δV1 Prevents Up-Regulation of L-Type Channels by Ethanol.

Cell Line	$^{45}Ca^{2+}$ Uptake, % Above Control	DHP Binding, % Above Control
PC12	56 ± 6	52 ± 18
C	52 ± 5	63 ± 7
V1δ2	−2 ± 12*	−20 ± 10*
V1δ3	15 ± 12*	−2 ± 10*
V1ε1	56 ± 6	35 ± 6
V1ε2	70 ± 13	41 ± 3

Note: PC12 cells were cultured for 6 days on polylysine-treated dishes in the absence or presence of 150 mM ethanol. Depolarization-induced $^{45}Ca^{2+}$ uptake and binding of (+)-$[^3H]$PN200-110 were measured as described (Gerstin et al. 1997) in PC12 cells, vector-transfected cells (C), and cells expressing the δV1 fragment (V1δ2, V1δ3) or the εV1 fragment (V1ε1, V1ε2). Data are mean ± SE values ($n = 5$–24). * $p < 0.05$ compared with PC12 by one-factor analysis of variance and Newman-Keuls post hoc test. DHP = dihydropyridine.

processes. PKCε is expressed predominantly in the nervous system and is particularly abundant in the hippocampus, olfactory tubercle, and layers I and II of cerebral cortex (Saito et al. 1993). Within immunoreactive neurons, it is localized to the Golgi apparatus, and to axons and presynaptic nerve terminals (Saito et al. 1993). PKCε is activated by growth factors that stimulate neural differentiation such as insulin (Heidenreich et al. 1990) and nerve growth factor (NGF) (Ohmichi et al. 1993). It is also the major PKC isozyme found in nondividing, differentiating neurons of the developing chick brain (Mangoura et al. 1993).

Our studies have provided evidence that PKCε plays an important role in modulating responses to NGF. PC12 cells are derived from neural crest and, when treated with NGF or fibroblast growth factors, undergo dramatic biochemical and morphological differentiation, developing several characteristics of mature sympathetic neurons (Greene et al. 1991). We found that ethanol enhances NGF-induced signal transduction leading to activation of Erk1 and Erk2 mitogen-activated protein (MAP) kinases (Roivainen et al. 1995) and NGF-induced neurite outgrowth (Roivainen et al. 1993). This effect is mimicked by PKC-activating phorbol esters (Roivainen et al. 1993) and is inhibited by depleting cells of PKC isozymes through prolonged treatment with high concentrations of phorbol ester (Roivainen et al. 1993). These results suggest that ethanol-induced increases in NGF responses are mediated by PKC.

Since ethanol up-regulates PKCδ and PKCε (Messing et al. 1991*b*), we

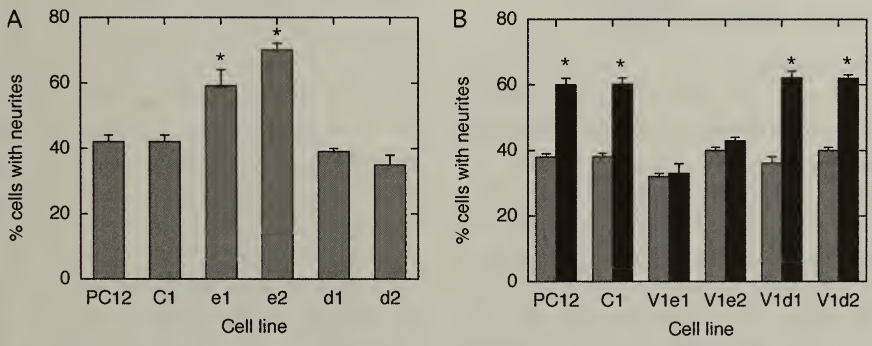

Figure 2. Protein kinase C epsilon (PKCε) enhances nerve growth factor (NGF)–induced neurite outgrowth and mediates enhancement of neurite outgrowth by ethanol. (A) The percentage of cells expressing neurites greater than one cell body in length was quantified in PC12 cells, vector-transfected cells (C1), and cells overexpressing PKCε (e1, e2), or PKCδ (d1, d2) after culture in 50 ng/mL of NGF for 4 days . $^*p < 0.05$ compared with PC12 cells and C1 cells by analysis of variance (ANOVA) and Scheffé F test. (B) The percentage of cells expressing neurites was quantified in cultures of PC12 cells, vector-transfected cells (C1), and cells expressing εV1 (V1e1, V1e2) or δV1 (V1d1, V1d2) following treatment with 50 ng/mL of NGF for 4 days in the presence (black bars) or absence (gray bars) of 100 mM ethanol. $^*p < 0.05$ compared with cells cultured without ethanol by ANOVA and Scheffé F test.

examined whether one of these two isozymes modulates responses to NGF. Using PC12 cell lines that stably overexpress these isozymes (Hundle et al. 1995), we found that overexpression of PKCε, but not of PKCδ, enhances NGF-induced MAP kinase activation (Hundle et al. 1995) and neurite outgrowth (figure 2A). In addition, we examined PC12 cell lines that stably express the fragments εV1 or δV1 and found that NGF-induced MAP kinase phosphorylation (Hundle et al. 1997) and neurite outgrowth were not enhanced by ethanol in cells expressing εV1, but were increased by ethanol in cells expressing δV1 (figure 2B). These findings establish PKCε as a positive modulator of neurite growth in PC12 cells and demonstrate that PKCε specifically mediates enhancement of responses to NGF by ethanol.

DISCUSSION

Up-regulation of L-type Ca^{2+} channels may contribute to the intense neuronal hyperexcitability observed during ethanol withdrawal, since L-type channel antagonists reduce tremors, seizures, and mortality in ethanol-dependent rodents deprived of ethanol (Little et al. 1986; Bone et al. 1989). Increases in L-type channels may also promote ethanol consumption, since withdrawal is associated with intense drug craving (Goldstein 1994), and L-type channel antagonists reduce ethanol consumption in animals (Rezvani and Janowsky 1990; Rezvani et al. 1991; Fadda et al. 1992; Colombo et al. 1994). It will be of interest to determine whether related voltage-gated Ca^{2+} channels are also up-regulated by ethanol and whether this requires activation of PKCδ. In addition, future studies with transgenic mice that overexpress and mutant mice that lack PKCδ will help determine whether PKCδ mediates up-regulation of L-type channels by ethanol in vivo and promotes the development of ethanol dependence.

Addiction appears to result from drug-induced changes in brain neurons following repeated drug exposure (Nestler and Aghajanian 1997). Such changes may include alterations in the structure of neuronal processes and remodeling of synaptic connections. Consistent with this possibility is the recent finding of increased dendritic length and density of dendritic spines in rat nucleus accumbens and prefrontal cortex after 5 weeks of repeated amphetamine exposure (Robinson and Kolb 1997). Our finding that ethanol-induced neurite outgrowth in vitro is mediated by PKCε suggests that this PKC isozyme plays an important role in ethanol-induced neural plasticity. Such plasticity is not limited to PC12 cells, since several reports indicate that ethanol enhances the growth of neural processes and terminals in specific brain regions in animals (reviewed in Messing et al. 1991*a* and Hundle et al. 1995). It will be an important challenge in future work to examine whether such structural changes contribute to addiction, and if they and associated addictive behaviors are reduced by inhibiting PKCε.

PKC has been implicated in so many cellular functions that it has been difficult to imagine how it could be usefully manipulated to treat disease. However, it is now clear that PKC is a family of isozymes, each with a unique structure, pattern of expression, and set of functions. Our studies demonstrate that it is possible to identify PKC-isozyme-specific events in situations where multiple isozymes are activated. Such research could lead to development of specific drugs targeted against PKCδ or PKCε to treat alcoholism. Experimental success with a high-affinity, specific oral inhibitor of PKCβ for treatment of diabetic vascular disease attests to the validity of such an isozyme-targeted approach (Ishii et al. 1996).

In summary, our in vitro studies indicate that chronic exposure to ethanol increases the abundance of two PKC isozymes, PKCδ and PKCε; figure 3 presents a model of PKC-isozyme-specific responses to chronic ethanol exposure. Increases in PKCδ appear important for up-regulation of L-type voltage-gated Ca^{2+} channels by ethanol, whereas increases in PKCε promote NGF-induced neurite outgrowth. The mechanisms by which ethanol induces

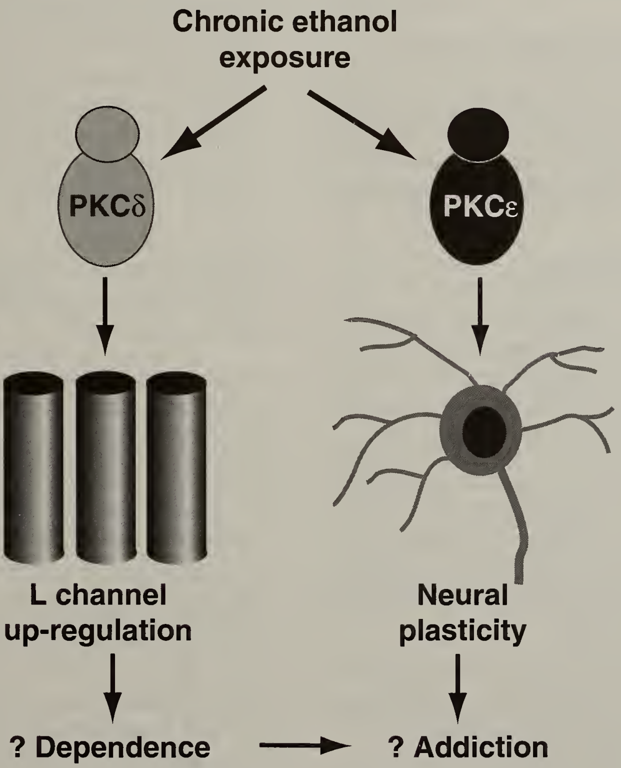

Figure 3. Model of PKC isozyme–specific responses to chronic ethanol exposure, indicating the relationships between PKCδ and PKCε to L-type channel up-regulation and neurite growth.

these two isozymes, and the mechanisms by which they, in turn, alter Ca^{2+} channel function and neuronal structure, remain topics of active investigation. Up-regulation of L-type channels appears to mediate certain manifestations of ethanol dependence, whereas enhancement of neurite outgrowth might be important for neural plasticity associated with addiction. It is hoped that future studies with transgenic mice will allow us to understand the relationships between these ethanol-induced biochemical and structural changes and ethanol-related behaviors.

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Chapter 13

Regulation of Ethanol Sensitivity in *Drosophila* by the Neuropeptide Gene *amnesiac*

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and Ulrike Heberlein, Ph.D.

Key words: biological regulation; AOD (alcohol or other drug) sensitivity; neuropeptides; genetic screening method; cAMP; biological activation; cell signaling; animal study

The fruit fly *Drosophila* is one of the most widely used and successful model systems in the study of development and behavior. Research in these fields has clearly demonstrated that the genes and biochemical pathways have been conserved during evolution, and many genes first identified in *Drosophila* have provided major insights into vertebrate and human development and disease. Flies have a relatively sophisticated nervous system and are capable of many complex behaviors.

The natural environment of *Drosophila melanogaster* includes fermenting plant materials, which often contain high levels of ethanol and other alcohols (3 percent or more) (McKechnie and Morgan 1982; Van Delden 1982). *Drosophila* is quite resistant to the toxic effects of ethanol and can degrade ethanol efficiently for use as an energy source or substrate for lipid biosynthesis (reviewed in Geer et al. 1993). The enzyme alcohol dehydrogenase (ADH) plays a key role in

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ensuring both metabolic use and detoxification from ethanol.

We have found that the behavior of flies upon exposure to ethanol is remarkably similar to that of inebriated humans and rodents. In addition, many of the neurotransmitter systems and signal transduction cascades, whose function is altered by ethanol in mammalian cells or tissues, have been identified in *Drosophila.* To learn about the mechanisms that regulate ethanol sensitivity and to search for potential ethanol targets, we initiated a genetic screen for *Drosophila* mutants that respond abnormally to an acute ethanol exposure. One mutant isolated due to its increased sensitivity to ethanol-induced loss of postural control was found to be an allele of the memory mutant *amnesiac* (*amn*) (Quinn et al. 1979). The *amn* gene encodes a putative neuropeptide believed to act through adenylate cyclase (AC) to increase cyclic adenosine monophosphate (cAMP) levels (Feany and Quinn 1995). Using a combination of genetic and pharmacological approaches, we showed that proper regulation of the cAMP signaling pathway is important in establishing ethanol sensitivity in *Drosophila.* This signaling pathway has been implicated previously in the cellular response to ethanol (for a review, see Diamond and Gordon 1997).

RESULTS

We observed the behaviors of adult *Drosophila* during and after exposure to ethanol vapor. Within minutes of exposure to ethanol, flies become hyperactive, displaying a fivefold increase in locomotion. Activity is maximal between 5 and 10 minutes of exposure. After about 15–20 minutes, flies progress to a sedated state (reflected as a decrease in general locomotion); they begin to fall and eventually fail to right themselves. If ethanol is removed at this stage, the flies recover in approximately 10–15 minutes. Flies carrying lesions in the *Adh* gene, thereby metabolizing alcohol very slowly, progress through the same initial phases of intoxication as do wild-type flies but require several hours, rather than minutes, to recover.

We used an inebriometer to measure ethanol-induced loss of postural control in a population of flies. This apparatus, which was originally designed for selective breeding experiments (Cohan and Hoffman 1986; Weber 1988), is a 125-cm long vertical glass column containing a series of sloping mesh baffles on which flies can stand (figure 1A). Flies are introduced into the top of the column; upon exposure to ethanol vapor, flies lose postural control and begin to fall through the column. They are collected as they "elute" from the column and are counted. The elution profile of wild-type flies follows a normal distribution; the mean elution time (MET), approximately 20 minutes at our standard ethanol vapor concentration, is inversely proportional to their sensitivity to ethanol. The concentration of ethanol in the heads of flies at the time of elution from the column (severe intoxication) is approximately 35 mM. The degree of loss of postural control in the inebriometer is dependent on the concen-

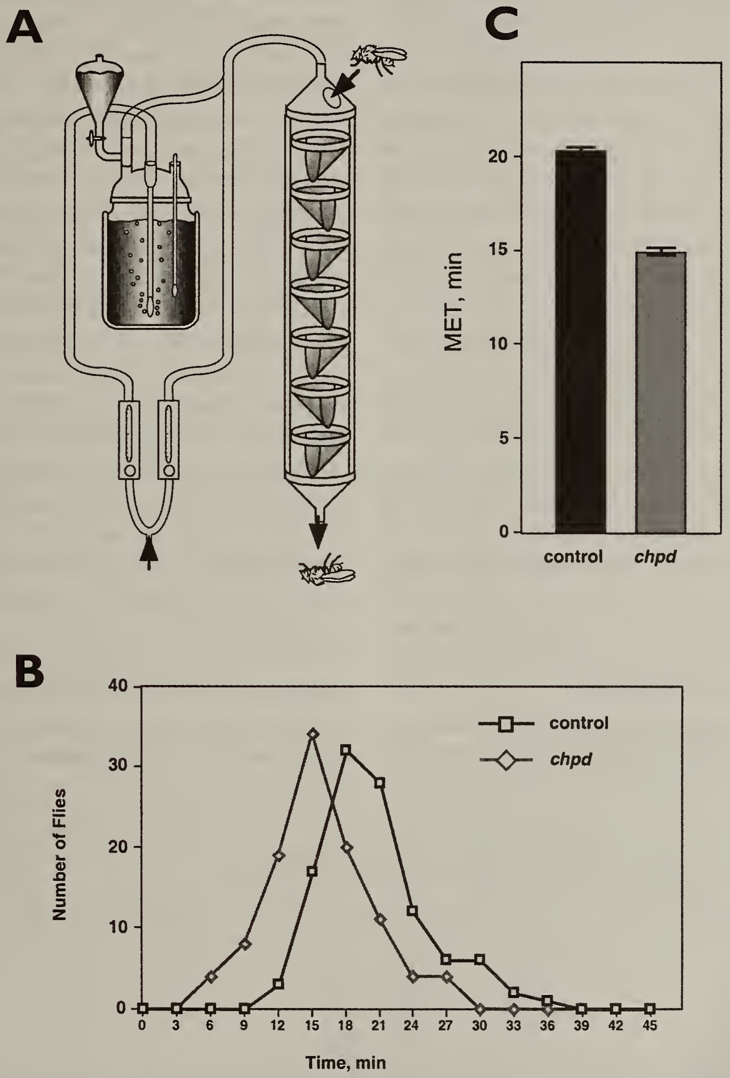

Figure 1. (A) Cartoon of an inebriometer. (B) Representative inebriometer elution profile of *chpd* and control flies. Approximately 100 male *chpd* or control flies were exposed to ethanol vapor in the inebriometer. The number of *chpd* or control flies eluting from the inebriometer as a function of time (minutes) is shown. (C) Average mean elution time (MET) of *chpd* and control males. The MET of *chpd* is significantly lower than that of control flies ($p < 0.0001$; $n = 31$). Reprinted with permission from Moore, M.S.; Dezazzo, J.; Luk, A.Y.; Tully, T.; Singh, C.M.; and Heberlein, U. Ethanol intoxication in *Drosophila:* Genetic and pharmacological evidence for regulation by the cAMP pathway. *Cell* 93:997–1007, 1998. Copyright 1998 by Cell Press.

tration of ethanol; reasonable dose-response curves can thus be obtained.

A genetic screen was carried out to isolate P element–induced mutants with altered sensitivity to ethanol intoxication using the inebriometer as the behavioral assay. One X-linked mutation isolated in this screen was named *cheapdate* (*chpd*) due to the increased ethanol sensitivity displayed by hemizygous mutant male flies. As shown in figure 1 (parts B and C), *chpd* males elute from the inebriometer with a MET of 15 minutes, compared with 20 minutes for the wild-type controls. This increased ethanol sensitivity of *chpd* males was observed at all ethanol vapor concentrations tested. The fact that *chpd* flies display normal ethanol absorption and metabolism indicates that the mutation does not alter ethanol pharmacokinetics (Moore et al. 1998).

The *chpd* P element insertion was mapped cytologically to band 19A1-2 on the X chromosome, a location to which *amnesiac* had been mapped previously (Tully and Gergen 1986). The *amn* gene was first isolated in a genetic screen for mutants with associative memory defects (Quinn et al. 1979). To determine whether *chpd* was an allele of *amn*, we tested additional *amn* alleles for ethanol sensitivity in the inebriometer. Hemizygous males carrying the original ethyl methanesulfonate (EMS)–induced *amn*^{1} allele or the *amn*^{X8} deletion allele also showed an ethanol-sensitive phenotype (Moore et al. 1998). More importantly, when assayed in females, *amn*^{1} and *amn*^{X8} failed to complement the ethanol sensitivity of *chpd* (table 1). These data demonstrate that *chpd* is an allele of *amn* and that the *amn* locus is involved in controlling ethanol sensitivity.

The *amn* gene was cloned previously and shown to encode a novel protein with weak homology to two mammalian neuropeptides: pituitary adenylyl cyclase activating peptide

Table 1. Mean Elution Time (MET) of *Drosophila* Genotypes.

Genotype	**MET ± SEM (min)**	***n***
+/+	20.22 ± 0.08	6
chpd/+	19.90 ± 0.18	6
chpd/*chpd*	15.33 ± 0.08	5
amn^{1}/+	20.01 ± 0.21	6
amn^{1}/*amn*^{1}	15.47 ± 0.09	6
amn^{x8}/+	19.66 ± 0.11	4
amn^{x8}/*amn*^{x8}	11.76 ± 0.19	4
amn^{1}/*chpd*	15.63 ± 0.11	8
amn^{x8}/*chpd*	15.58 ± 0.07	4

Note: Genetic complementation tests between *chpd*, *amn*^{1}, and *amn*^{x8} were performed by assaying virgin females. All *amn* alleles and *chpd* are completely recessive; *amn*^{1} and *amn*^{X8} failed to complement the ethanol sensitivity of *chpd* (n = 4). Reprinted with permission from Moore, M.S.; Dezazzo, J.; Luk, A.Y.; Tully, T.; Singh, C.M.; and Heberlein, U. Ethanol intoxication in *Drosophila:* Genetic and pharmacological evidence for regulation by the cAMP pathway. *Cell* 93:997–1007, 1998. Copyright 1998 by Cell Press.

(PACAP) and growth hormone–releasing hormone (GHRH) (Feany and Quinn 1995), both of which are known to activate receptors that couple positively with AC to increase cAMP levels. We recovered and sequenced approximately 1.1 kb of the genomic DNA flanking the *chpd* P element insertion site. A search of the National Center for Biotechnology Information database revealed that the P element had inserted within the open reading frame of the *amn* gene C-terminal to the PACAP and GHRH homology regions (figure 2). On the basis of the genetic and molecular data shown above, *chpd* will henceforth be referred to as amn^{chpd}.

Genetic data have implicated *amn* in activation of the cAMP pathway (Feany and Quinn 1995). To further explore a potential role of the cAMP pathway in regulating ethanol sensitivity, we extended our behavioral analysis to flies carrying mutations in molecules involved in cAMP signaling: (1) *rutabaga* (*rut*), encoding the Ca^{2+}-calmodulin-sensitive AC (Livingstone et al. 1984; Levin et al. 1992), and (2) *dunce* (*dnc*), encoding the major cAMP-phosphodiesterase (Chen et al. 1986; Qui et al. 1991). Males hemizygous for rut^{1} or rut^{769} (Livingstone et al. 1984; Han et al. 1992) displayed an ethanol-sensitive phenotype similar to that of *amn* mutants (figure 3). Ethanol sensitivity of males hemizygous for the dnc^{1} or dnc^{M11} mutations (Dudai et al. 1976; Mohler 1977), however, was nearly normal. These data show that flies unable to increase cAMP levels normally (such as *rut* and possibly *amn*) are more sensitive to ethanol-induced loss of postural control. The converse, however, is not observed; *dnc* flies, whose cAMP levels are several times higher than wild-type flies (Byers et al. 1981; Davis and Kiger 1981), display nearly normal ethanol sensitivity.

We also used the AC activator forskolin to manipulate cAMP levels in adult flies. Control and amn^{chpd} males were fed a 10-µM forskolin solution for 2 or 4 hours before assaying their ethanol sensitivity in the inebriometer. Whereas forskolin treatment had no effect on the behavior of control flies, the ethanol sensitivity defect of amn^{chpd} flies was reversed by a 2-hour forskolin treatment (figure 4). Interestingly, a 4-

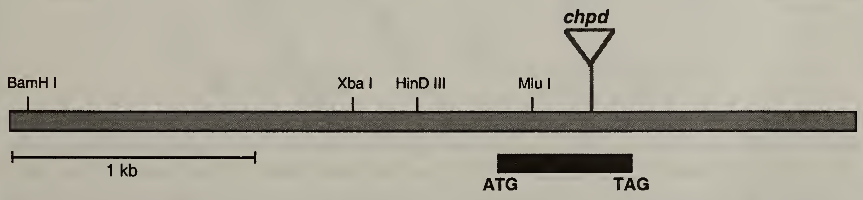

Figure 2. A genomic map of the *amn* locus displaying the location of the P element insertion in *chpd* (triangle). Reprinted with permission from Moore, M.S.; Dezazzo, J.; Luk, A.Y.; Tully, T.; Singh, C.M.; and Heberlein, U. Ethanol intoxication in *Drosophila:* Genetic and pharmacological evidence for regulation by the cAMP pathway. *Cell* 93:997–1007, 1998. Copyright 1998 by Cell Press.

hour forskolin treatment of *amn*^{chpd} males further reduced ethanol sensitivity, suggesting that one or more components of the cAMP pathway may have undergone compensatory up-regulation in *amn*^{chpd} mutants, thereby increasing the system's ability to respond to pharmacologically induced increases in cAMP levels. Taken

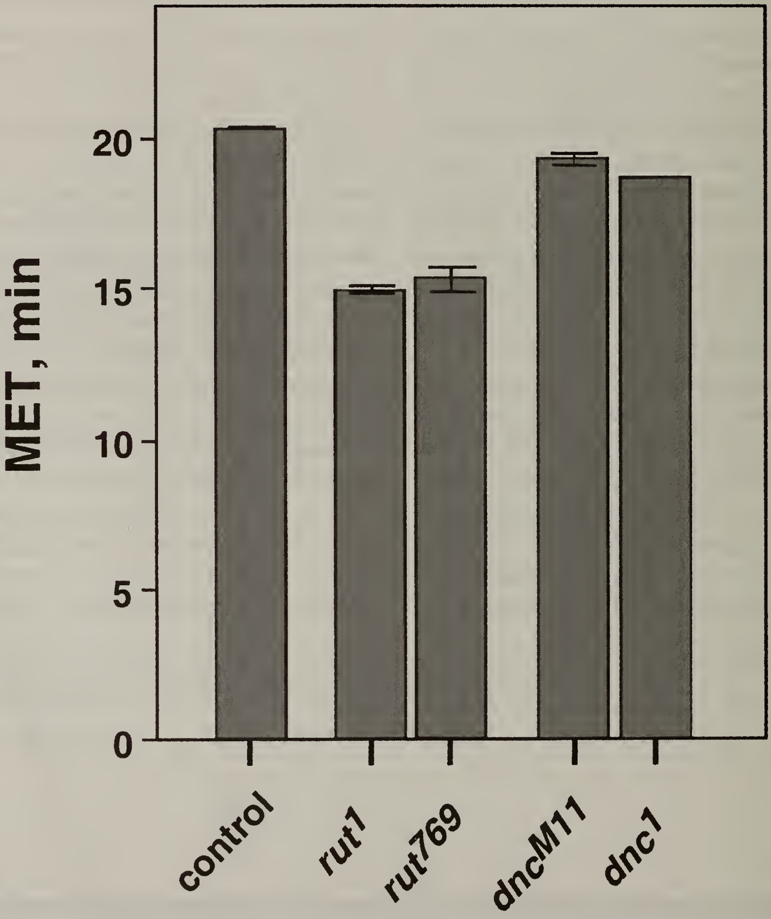

Figure 3. Mean elution time (MET) of mutations that impair cyclic adenosine monophosphate metabolism. Previously characterized alleles of *rut* (the loss-of-function EMS-induced *rut*^{1} allele and P element–induced allele *rut*^{769}) were significantly more sensitive to ethanol vapor compared with control flies ($p < 0.0005$; $n = 4$). The MET of *dnc*^{1}, but not that of *dnc*^{M11}, was significantly different from the MET of control flies ($p < 0.0001$ and $p = 0.028$, respectively; $n = 4$). Reprinted with permission from Moore, M.S.; Dezazzo, J.; Luk, A.Y.; Tully, T.; Singh, C.M.; and Heberlein, U. Ethanol intoxication in *Drosophila:* Genetic and pharmacological evidence for regulation by the cAMP pathway. *Cell* 93:997–1007, 1998. Copyright 1998 by Cell Press.

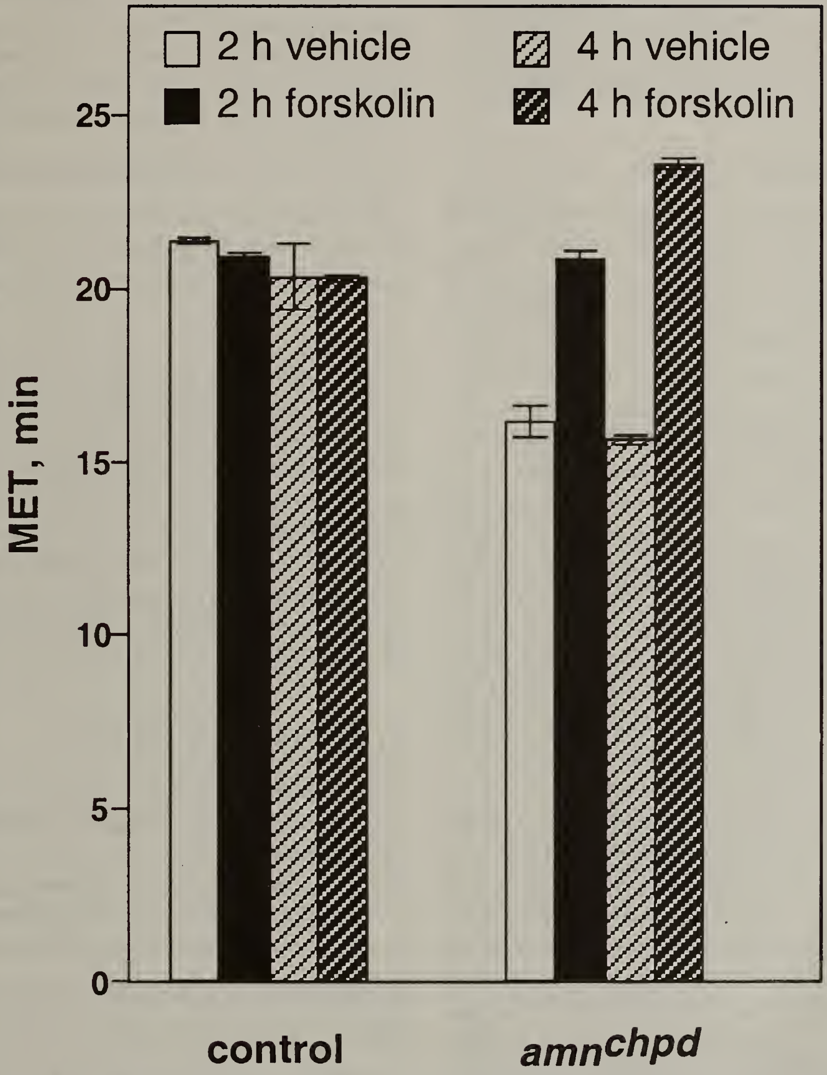

Figure 4. Effect of forskolin treatment on ethanol sensitivity in adult flies. Control and amn^{chpd} male flies were fed a sucrose solution (vehicle) or the same solution containing 10 μM forskolin for 2 or 4 hours prior to being assayed in the inebriometer. Treatment with forskolin for 2 hours significantly increased the MET of amn^{chpd} flies ($p < 0.0001$; $n = 4$) but had little effect on control flies ($p = 0.07$; $n = 4$). The MET of amn^{chpd} and that of control flies treated with forskolin for 4 hours was, however, significantly different ($p < 0.0001$; $n = 5$). Reprinted with permission from Moore, M.S.; Dezazzo, J.; Luk, A.Y.; Tully, T.; Singh, C.M.; and Heberlein, U. Ethanol intoxication in *Drosophila:* Genetic and pharmacological evidence for regulation by the cAMP pathway. *Cell* 93:997–1007, 1998. Copyright 1998 by Cell Press.

together, these data suggest that the effects of *amn* and *rut* on ethanol sensitivity are directly related to their ability to modulate cAMP levels.

CONCLUSIONS

In a screen for *Drosophila* mutants with abnormal responses to an acute ethanol exposure, we isolated *chpd*, a mutant with increased sensitivity to ethanol-induced loss of postural control. We demonstrated that *chpd* is a mutation in the *amn* gene, which is believed to encode a neuropeptide that stimulates cAMP production (Feany and Quinn 1995).

In mammalian cells and tissues, ethanol potentiates receptor-mediated cAMP signal transduction (for a review, see Gordon et al. 1992); the mechanisms underlying this effect, however, remain poorly understood. Although a direct link between cAMP signaling and ethanol-induced behaviors has not been established in mammals, the responses to acute ethanol are thought to be mediated by alterations in the function of various ligand-gated ion channels. Certain subtypes of gamma-aminobutyric acid type A ($GABA_A$) and *N*-methyl-D-aspartate (NMDA) receptors are potentiated and inhibited by ethanol, respectively (for reviews, see Grant and Lovinger 1995; Buck 1996; Crews et al. 1996), and both these channels can be phosphorylated by protein kinase A (PKA) in cells, tissues, or heterologous expression systems (for a review, see Tabakoff and Hoffman 1996). It is tempting to speculate that PKA-phosphorylation of neurotransmitter receptors is altered by ethanol and that this contributes to the behavior of the inebriated animal.

In summary, we have shown that proper activation of the cAMP pathway plays an important role in regulating ethanol sensitivity in flies. This signaling pathway has been implicated previously in regulating ethanol responses in mammalian cells (Diamond and Gordon, 1997). Moreover, the levels of AC are frequently reduced in lymphocytes and platelets obtained from alcoholic subjects, even after long periods of abstinence (Diamond et al. 1987; Tabakoff et al. 1988). *Drosophila* may thus provide a powerful genetic model in which to identify relevant novel genes that control ethanol sensitivity and neural responses to alcohol. Since resistance to ethanol has been correlated with alcoholism in humans (Schuckit 1994), genes and pathways identified in *Drosophila* may aid the study of the mechanisms responsible for this phenotype.

ACKNOWLEDGMENTS

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Chapter 14

Ethanol Sensitivity and Molecular Function of Fyn Tyrosine Kinase

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KEY WORDS: AOD (alcohol or other drug) sensitivity; molecular genetics; tyrosine; protein kinases; spatial perception; aggressive behavior; AODR (AOD related) seizure; central nervous system; synapse; AOD tolerance

Ethanol is among the most widely abused drugs in the world, but the neural mechanisms responsible for ethanol intoxication and dependence are unknown. Gene knockout and quantitative trait locus (QTL) analyses are revealing that genetic factors affect the determination of the behavioral responses to ethanol in rodents. In the gene knockout analyses, it has been demonstrated that loss of a single gene in mice can cause defects in ethanol sensitivity. Disruption of a gene encoding each of the synaptic molecules—protein kinase Cγ (PKCγ), gamma-aminobutyric acid A ($GABA_A$) receptor, and dopamine receptor—affects the sensitivity to ethanol (Harris et al. 1995; Homanics et al. 1997; Rubinstein et al. 1997). Another powerful approach using mouse genetics is QTL, which takes advantage of recombinant inbred mouse strains (Plomin and McClearn 1993; Crabbe et al. 1994). The DBA/2 strain is well known for its behavioral abnormalities and has been extensively studied, especially in drug sensitivity, audiogenic seizures, and learning defect, particularly in comparison with behaviors of the C57BL/6 strain (Blum et al. 1983; Upchurch and Wehner 1989; Neumann and Collins 1991). The QTL analysis of BXD recombinant inbred mouse strains, which are fully inbred descendants of

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an F2 intercross between C57BL/6 and DBA/2 strains, have revealed multiple genes underlying ethanol sensitivity observed in the DBA/2 strain.

Fyn, which belongs to the Src family of protein tyrosine kinases, has a crucial molecular function in determining patterns of behavior. Loss of Fyn function in mice is related to several behavioral defects, such as spatial learning, as tested in the Morris water maze (Grant et al. 1992), suckling behavior (Yagi et al. 1993), hyperresponsiveness to fear-inducing stimuli (Miyakawa et al. 1994), and enhanced susceptibility to audiogenic seizures (Miyakawa et al. 1995). Fyn contributes to the induction of *N*-methyl-D-aspartate (NMDA)–dependent long-term potentiation (LTP) in the hippocampus (Grant et al. 1992) and olfactory bulb (Kitazawa et al. 1998). These defects suggest that Fyn plays critical roles in determining mammalian behavior. In the adult neuron, Fyn is concentrated in the postsynaptic density (PSD) fraction (Grant et al. 1992; Kohmura et al. 1998). Recently, upstream of Fyn in the synapse, we have identified a diverged cadherin-related neural receptor (CNR) family by the yeast two-hybrid system (Kohmura et al. 1998) and p130 (Yasuda and Yagi 1997).

SENSITIVITY OF FYN-DEFICIENT MICE TO ETHANOL

We focused on behavioral similarities between Fyn-deficient and DBA/2 mice, including defects of spatial learning in the Morris water maze, aggressive behavior, and enhanced susceptibility to audiogenic seizure (table 1). Because of these similarities, and because the DBA/2 strain is more sensitive to ethanol than is the C57BL/6 strain, we hypothesized that Fyn is a candidate for determining sensitivity to ethanol. Supporting this hypothesis, tyrosine kinases, including Fyn, phosphorylate NMDA and $GABA_A$ receptors and modulate the electrophysiological function of these receptors (Wang and Salter 1994; Suzuki and Okumura-Noji 1995; Valenzuela et al. 1995; Moss et al. 1997; Yu et al. 1997). The function of these receptors is modulated by ethanol, and they are hypothesized to be targets through which ethanol exerts its behavioral effects (Lovinger et al. 1989, 1990; Wafford et al. 1990).

When we assessed the hypnotic effect of ethanol on Fyn-deficient mice (Miyakawa et al. 1997), the duration of the loss of righting reflex (LORR) after ethanol administration was significantly longer for Fyn-deficient mice than for

Table 1. Similar Behavioral Phenotypes Between DBA/2 Strain and Fyn-Deficient Mice.

Behavior	DBA/2	Fyn-Deficient
Spatial learning in Morris water maze	Impaired	Impaired
Emotional behavior	Abnormal	Abnormal
Audiogenic seizure	Quite susceptible	Susceptible
Ethanol sensitivity	Sensitive	Sensitive

control, heterozygous mice. When we administered flurazepam (a benzodiazepine derivative) to induce the similar LORR with ethanol, no significant difference was found in the duration of the LORR at any flurazepam dose. Analysis of the blood ethanol concentration curve revealed no significant differences between Fyn-deficient and control mice. Therefore, the enhanced sensitivity to ethanol is likely due to changes in the sensitivity of the central nervous system rather than to changes in pharmacokinetic or metabolic factors. Thus, lack of Fyn tyrosine kinase increases the sensitivity to ethanol.

EFFECT OF ETHANOL ADMINISTRATION ON TYROSINE PHOSPHORYLATION OF NMDA RECEPTORS IN THE HIPPOCAMPUS

To find the molecular mechanism of Fyn tyrosine kinase for determining the sensitivity to ethanol, we examined tyrosine-phosphorylated proteins by administration of ethanol in the brain. It is known that ethanol enhances tyrosine phosphorylation in a human epidermal carcinoma (Thurston and Shukla 1992) and in neural cell line PC12 cells (Roivainen et al. 1995). After saline or ethanol (3.5 g/kg) administration for Fyn-deficient or control mice, we dissected cerebral cortex, hippocampus, and cerebellum and obtained the protein extracts from these brain regions. The level of tyrosine phosphorylation after saline treatment was not significantly different between Fyn-deficient and control mice. A significant enhancement in tyrosine phosphorylation of a 180-kd protein 5 minutes after ethanol administration was observed in hippocampus of the control mouse but not in hippocampus of the Fyn-deficient mouse. Interestingly, this tyrosine phosphorylation of 180-kd protein was only observed in hippocampus but not in cerebral cortex and cerebellum. The lack of this increase in Fyn-deficient mouse indicated that its tyrosine phosphorylation of 180-kD protein is mainly mediated by Fyn tyrosine kinase.

In adult brain, Fyn is enriched in the PSD fraction (Grant et al. 1992). NMDA receptor (NMDAR) 2A (corresponding to NMDARε1 in mice) and NMDAR2B (corresponding to NMDARε2 in mice) is found as a protein band of molecular mass 180 kd in the PSD fraction. In addition, these receptors are colocalized with Fyn in the PSD and phosphorylated by Fyn (Kohmura et al. 1998). Consequently, we investigated the level of tyrosine phosphorylation of NMDARε1 and NMDARε2 after ethanol administration in the hippocampus, where the increase occurred. Enhanced tyrosine phosphorylation of NMDARε2 5 minutes after ethanol administration was observed in the control mice but not in Fyn-deficient mice. On the other hand, a similar amount of tyrosine phosphorylation was found between the control and Fyn-deficient mice. As concerns NMDARε1, we could not find enhancement of tyrosine phosphorylation by the ethanol administration. It is therefore likely that the enhanced tyrosine phosphorylation of NMDARε2 after ethanol administration accounts for the enhancement of tyrosine phosphorylation of 180-kd protein. The tyrosine phosphorylation of NMDARε2 is also observed in response to lesions with 6-OH-dopamine (Menegoz et al. 1995), taste learning (Rosenblum et al. 1997), and the induction of LTP (Rosenblum et al. 1996; Rostas et al. 1996). In ethanol administration, we can conclude that tyrosine phosphorylation of NMDARε2 is mediated by Fyn tyrosine kinase.

INDUCTION OF ACUTE TOLERANCE TO ETHANOL WITH TYROSINE PHOSPHORYLATION OF NMDARε2 BY FYN

The inhibition of NMDAR-mediated excitatory postsynaptic potentials (EPSPs) by ethanol is gradually reduced during the period of ethanol exposure in hippocampal slices (acute tolerance). This acute tolerance might be caused by the up-regulation of tyrosine phosphorylation of NMDARε2, because NMDAR currents are potentiated by tyrosine kinase in the hippocampal neurons (Wang and Salter 1994; Yu et al. 1997). To test this possibility, we compared the effects of ethanol on NMDA-mediated EPSPs in the CA1 hippocampal neurons of the control and Fyn-deficient mice. Bath application of ethanol initially suppressed NMDA-mediated EPSPs, but the amplitude of the EPSPs gradually recovered in the control mice during the application of ethanol, showing the development of acute tolerance. By contrast, in Fyn-deficient mice, ethanol suppressed NMDA-mediated EPSPs with little sign of development of tolerance, and EPSPs recovered their original amplitude after ethanol was removed. Thus, modulation of NMDA function by Fyn seems to be involved in the development of acute tolerance to ethanol. Furthermore, the acute tolerance was eliminated when ethanol was applied together with ifenprodil, an agent considered to be a selective antagonist of NMDARε2 (Williams 1993). These findings are consistent with the notion that enhancement of tyrosine phosphorylation of NMDARε2 is a basis of the acute tolerance. Fyn-deficient mice showed abnormalities in their behavioral sensitivity to ethanol together with abnormal responses of NMDAR to ethanol.

SCREENING GENETIC FACTORS FOR ETHANOL SENSITIVITY BY FYN

We have characterized the molecular function of NMDAR and Fyn to determine the sensitivity to ethanol, but other genetic factors may exist for this regulation. An inbred mouse strain of DBA/2, which shows behavioral abnormalities similar to Fyn-deficient mice, is also sensitive to ethanol. We assumed that differential genetic components of regulating behavior in the DBA/2 strain were related to the signal transduction pathway of Fyn. To isolate molecules regulating behavior, we screened the disparate Fyn-binding proteins between C57BL/6 and DBA/2 mouse strains. The bulk of Fyn-binding protein purified with the GSTFynK-affinity column from the P2 fraction of C57BL/6 brains was immunized into mice, and various monoclonal antibodies were obtained. To select the proteins on regulating ethanol sensitivity, we performed immunoblotting analysis with each these antibodies against brain extracts from C57BL/6 and DBA/2 strains.

Interestingly, a monoclonal antibody recognized different-sized bands between C57BL/6 and DBA/2, 130 and 140 kd, respectively. We named both the 130-kd and 140-kd bands "p130." These different-sized proteins were derived from chromosomal alteration, but not from different posttranscriptional modification. The p130 was abundantly expressed in the brain and was extensively concentrated in the PSD fraction. When we performed immunoprecipitation using Fyn antibodies, immunocomplex prepared with Fyn antibodies contained p130 protein. These findings indicated that p130 was a candidate genetic component for regulating sensitivity to ethanol through the signal transduction pathway of Fyn at the synapse. The p130 was likely to be colocalized with microtubule (unpublished data). $GABA_A$ receptor is associated with microtubule (Item and Sieghart 1994) and has a binding site for ethanol (Mihic et al. 1997). Interestingly, microtubule depolymerization inhibits ethanol-induced enhancement of $GABA_A$ responses (Whatley et al. 1996). These data also supported the hypothesis that p130 was a candidate molecule to regulate ethanol sensitivity.

QTL analysis of mouse strains grossly confirmed chromosomal locus for regulating sensitivity to ethanol. Therefore, confirming the chromosomal locus of the p130 was significant for understanding which behaviors related to the difference of the p130 between C57BL/6 and DBA/2. Over 30 recombinant inbred strains between C57BL/6 and DBA/2 have been maintained as BXD strains. We used these strains for mapping the chromosomal locus of p130 protein by immunoblot analysis. Upon comparison with the strain distribution pattern of loci typed in BXD recombinant inbred strains (Item and Sieghart 1994), only the Hbb locus in chromosome 7 exhibited the same pattern of p130. These results indicate that the p130 locus is closely linked to the Hbb locus, which is located between Mod-2 and Odc-7 on mouse chromosome 7. QTL analysis revealed that gene loci for regulating ethanol sensitivity are located in chromosome 7 (Crabbe et al. 1994). Loss of Fyn also induces greater sensitivity to ethanol; therefore, the p130 bound with Fyn in PSD was the most promising gene for regulating the synaptic function on sensitivity to ethanol.

CONCLUSION

Fyn signal transduction in the neurons is essential to determine the ethanol sensitivity. Mice lacking Fyn show more sensitivity to ethanol. Under experimental conditions demonstrating enhanced sensitivity to ethanol in mice lacking Fyn, we confirmed the molecular mechanism by which Fyn phosphorylates to NMDARε2 after ethanol administration. Based on similarity of the ethanol sensitivity between Fyn-deficient and DBA/2 mice, we newly identified a different genetic component between C57BL/6 and DBA/2 mouse strains as p130, which is bound to Fyn and is enriched in the PSD fraction. Since p130 is likely to be colocalized to microtubule and functional $GABA_A$ receptor binds to microtubule at the synapse, we speculate that Fyn is linked to $GABA_A$ receptor function via p130 and microtubule complex. It is known that NMDA and $GABA_A$ receptors are related to ethanol sensitivity. We would like to propose that Fyn tyrosine kinase is a modulator between NMDA and $GABA_A$ receptors for determining ethanol sensitivity at the synapse (figure 1).

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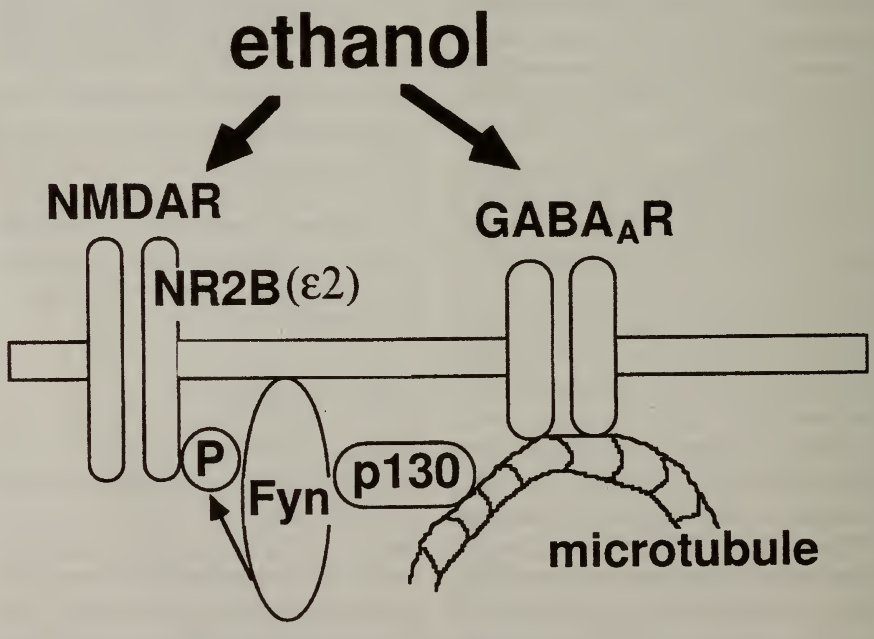

Figure 1. Molecular interactions of Fyn tyrosine kinase under postsynaptic membrane. Fyn can phosphorylate *N*-methyl-D-aspartate receptor NMDAR2B (ε2) after ethanol administration. Ethanol binds to gamma-aminobutyric acid A receptor ($GABA_AR$), whose function is influenced by microtubule stabilization. Fyn binding protein, p130, in postsynapse is colocalized with microtubule. Therefore, we speculated that Fyn also has reciprocal interaction with $GABA_AR$ function. P = tyrosine phosphorylation.

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Chapter 15

Effects of β-Endorphin Levels on Ethanol Self-Administration in Mice

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KEY WORDS: beta-endorphin; AODE (effects of AOD [alcohol or other drug] use, abuse, and dependence); self administration of drugs; reinforcement; endogenous opioids; AOD craving; AODD (AOD use disorder) relapse; AOD preference; dose response relationship; hypothesis testing

β-Endorphin, a member of the opioid peptide family, is an endogenous ligand for the metabotropic μ opioid receptor. This receptor, like other opioid receptors, is negatively coupled to adenlyl cyclase and thus upon activation results in decreased cyclic adenosine monophosphate (cAMP) production. The peptide β-endorphin derives from the proopiomelanocortin (POMC) gene along with adrenocorticotropic hormone (ACTH), β-lipotropin, α-melanocyte-stimulating hormone, and other related peptides. The primary sites of POMC biosynthesis are the anterior and intermediate lobes of the pituitary gland, the arcuate nucleus of the hypothalamus, and a small group of neurons in the nucleus tractus solitarii (Eipper and Mains 1980). The primary endorphinergic projections in the central nervous system are from the arcuate nucleus to brain regions involved in both reward (e.g., septum and nucleus accumbens [Wise and Bozarth 1982]) and stress (periaqueductal gray and amygdala [Khachaturian et al. 1985]). In fact, mice lacking β-endorphin are deficient in opioid-mediated stress-induced analgesia (Rubinstein et al. 1996). This chapter summarizes the rationale and findings for an investigation of β-endorphin's role in the reinforcing effects of ethanol.

OPIOIDS AND ALCOHOL

Endogenous opioids have been hypothesized to be an important component of

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the neural substrate for ethanol actions, and the relationship between ethanol and these peptides has been investigated for a number of years (see Herz 1997 for a review). To date, research on the specific relationship between ethanol and β-endorphin has taken one of two general strategies.

The first strategy has involved studying the β-endorphin response to ethanol, and much is currently known about these effects. For instance, acute ethanol administration increases β-endorphin synthesis and release, whereas chronic exposure to ethanol appears to have the opposite effects (Schulz et al. 1980; Keith et al. 1986; Gianoulakis 1990; Scanlon et al. 1992; De Waele and Gianoulakis 1993; Przewlocka et al. 1994). These changes are postulated to underlie some of the important behavioral consequences of ethanol, such as those resulting from dopamine release in the mesolimbic pathway (Widdowson and Holman 1992). Alcohol consumption, craving, and relapse have thus been linked to the endogenous opioid system and, by implication, to β-endorphin in particular (e.g., Gianoulakis et al. 1996; O'Brien et al. 1996; Davidson et al. 1996).

A genetic risk for alcoholism has been well established (e.g., Goodwin et al. 1973, Cloninger et al. 1981). Clearly polygenic, in recent years specific candidate genes have been postulated to contribute to this complex trait. Thus, the second general line of inquiry looks for differences in β-endorphin between populations of animals (including humans) that differ in their sensitivity to ethanol. Many studies have suggested that heritable differences in the opioid system may be related to alcoholism (e.g., Topel 1988; Gianoulakis et al. 1989; Aguirre et al. 1995, del Arbol et al. 1995; Herz 1997). One such hypothesis specifies a role for β-endorphin.

Several strains of rodents that differ in their preference for ethanol have been shown also to differ with respect to β-endorphin. For example, differences in the brain content of β-endorphin have been reported between AA rats (selectively bred to be alcohol preferring) relative to ANA rats (alcohol avoiding) as well as between inbred mouse strains that differ in voluntary consumption of ethanol (De Waele and Gianoulakis 1994; De Waele et al. 1994). Furthermore, the ethanol-induced rise in hypothalamic β-endorphin is more robust for ethanol-preferring C57BL/6 mice than ethanol-avoiding DBA/2 mice (De Waele et al. 1992; De Waele and Gianoulakis 1994).

The differences in β-endorphin observed in rodents that are correlated with ethanol consumption appear to hold for humans as well. Relative to nonalcoholics, lower plasma β-endorphin has been found in alcoholics (Vescovi et al. 1992; Aguirre et al. 1995; del Arbol et al. 1995) as well as those at high risk for the development of alcoholism (Gianoulakis et al. 1989). Moreover, Gianoulakis and colleagues (1995, 1996) found that nonalcoholic subjects from families with at least a two-generation history of alcoholism showed an enhanced dose-dependent increase in β-endorphin plasma levels following oral consumption of ethanol relative to those from families without a history of alcoholism. Froehlich and colleagues

(2000) confirmed that the β-endorphin rise in response to ethanol is a heritable trait.

Pharmacotherapeutic support for the contention that opioids influence ethanol self-administration comes from the large body of evidence indicating that ethanol drinking decreases following administration of opiate antagonists. This was first demonstrated in rodents (e.g., Altshuler et al. 1980; Froehlich et al. 1990) and subsequently confirmed in clinical trials (O'Malley et al. 1992; Volpicelli et al. 1992). The supposition here is that by blocking the effects of the ethanol-induced opioid release, naltrexone attenuates the pleasure associated with drinking. In a recent study by Gonzales and Weiss (1998), naltrexone blocked the ethanol-mediated dopamine increase in the nucleus accumbens. This finding supports a model in which endorphinergic neurons disinhibit dopamine activity in the mesolimbic "reward" pathway.

These studies, either examining β-endorphin changes in response to ethanol, or correlating β-endorphin activity with ethanol consumption, provide ample rationale for investigating the way in which β-endorphin might alter ethanol sensitivity. Fortunately, the mechanism for such investigations has recently been made available.

β-ENDORPHIN MUTANTS AND ETHANOL CONSUMPTION

In order to study the physiological roles of β-endorphin, our laboratory (Rubinstein et al. 1996) used the genetic approach of homologous recombination in embryonic stem cells to produce mutant mice that are unable to synthesize β-endorphin. Because β-endorphin is posttranslationally processed from the carboxyl-terminal amino acid region of a multifunctional precursor peptide (POMC), the introduction of a premature stop codon (point mutation) into the POMC gene results in normal processing of all POMC products in the mutant mice with the exception of β-endorphin. These knockout mice appear normal in many respects, such as fertility, birth weight, activity, and systemic morphine analgesia (Rubinstein et al. 1996).

The purpose of this investigation was to begin asking the question: how do varying levels of endogenous β-endorphin affect ethanol-dependent responses? We tested genetically homogenous mice that express either 100 percent, 50 percent, or 0 percent of normal β-endorphin levels for their preference and consumption of ethanol using a two-bottle, free choice paradigm. Our findings support the notion that availability of the endogenous peptide is one of the factors that contributes to oral self-administration of ethanol.

Experimental animals were born and reared at the animal care facilities of Oregon Health Sciences University and then transferred to the Portland VA Medical Center animal research facility after weaning. Mating pairs consisted of heterozygote (+/–) males crossed with (+/–) females. The offspring segregated into the three genotypes (+/+, +/–, and –/–) in a Mendelian ratio of 1:2:1 as predicted for a recessive allele. These derived from original F_1 hybrid mice (129/Sv, C57BL/6N) that had been backcrossed to C57BL/6J for seven generations and were thus nearly congenic on the C57BL background. Genotyping was performed on genomic DNA samples obtained from

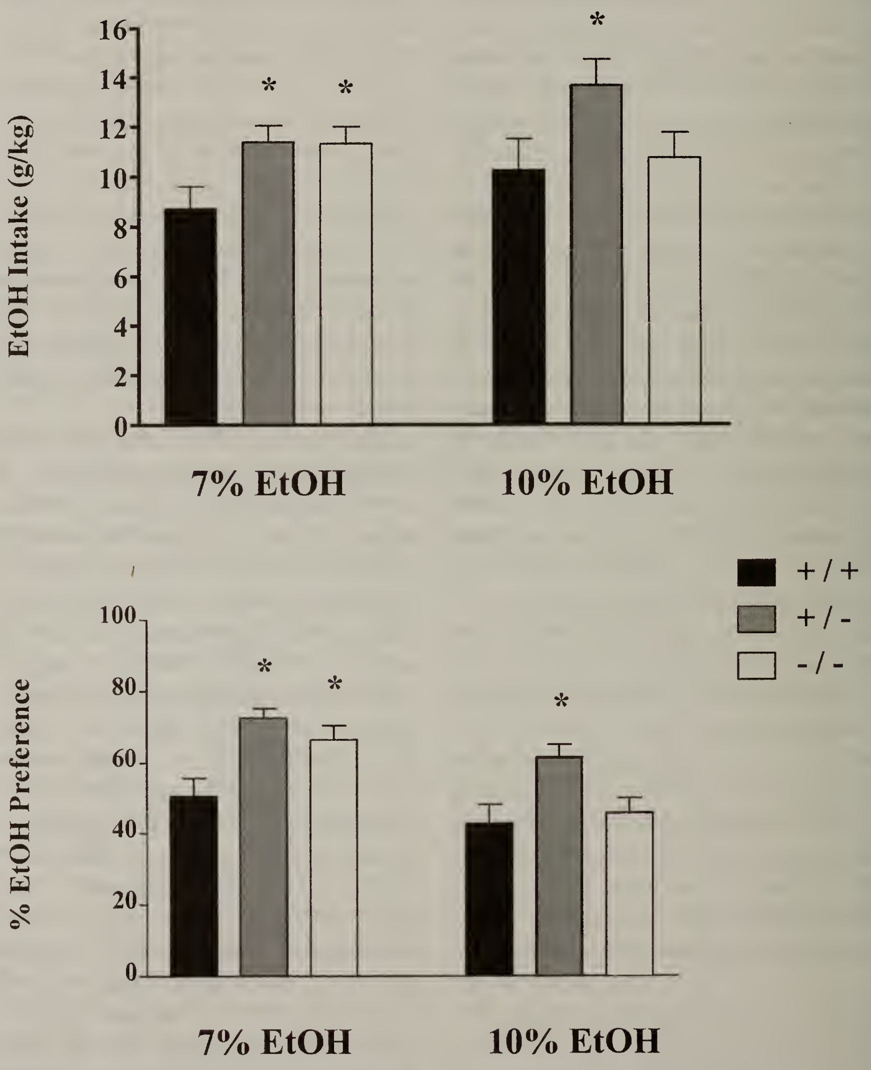

Figure 1. Ethanol (EtOH) consumption (top panel) and preference ratios (lower panel) for two-bottle choice of 7 percent and then 10 percent EtOH in wild-type (+/+), heterozygote (+/-), and homozygote β-endorphin-deficient (-/-) mice. Data represent the average drinking over each of the 4 day test periods at each dose, ± SEM. * Significantly different from wild-type mice. Data from Grisel et al. 1999.

mouse tails by polymerase chain reaction under standard conditions.

Male and female mice of each genotype were individually housed in a temperature- (21 ± 2 °C) and light-controlled (12L:12D) colony room. There were 117 test subjects, approximately equally divided by sex and genotype. Two fluid-filled 25-mL graduated cylinders were placed on each cage, food hoppers were filled with rodent block chow, and the tube volumes were recorded. Following 4 days of water drinking, one of the tubes (counterbalanced for side across cages) was filled with 7 percent ethanol for 8 days, and then 10 percent ethanol for 8 more days. Ethanol consumption was expressed as grams per kilogram per day (dividing the 48-hour consumption scores by 2), and preference ratios were calculated as the percent of total fluid intake consumed from the ethanol tube over that same period. Tube positions were changed every other day to dissuade the development of side preferences.

The results of this study are summarized in figure 1. Although the dose of self-administered ethanol did not significantly differ between genotypes ($F_{[2,107]} = 2.5$, $p < 0.1$ [top panel of the figure]), there was a main effect of dose ($F_{[1,107]} = 9.6$, $p < 0.01$) and, importantly, an interaction between genotype and dose ($F_{[2,107]} = 3.1$, $p < 0.05$). The three genotypes did differ significantly with respect to their overall preference for the ethanol solution ($F_{[2,114]} = 6.9$, $p < 0.01$ [lower panel of the figure]). The concentration of ethanol affected preferences ($F_{[1,114]} = 60.1$, $p < 0.001$), and there was an interaction between concentration and genotype, indicating that genotypes were not equally affected by the change in concentration ($F_{[2,114]} = 5.6$, $p < 0.01$). Taken together, these data reflect the fact that heterozygote mice consistently drank more ethanol than wild-type mice, while drinking in the knockouts was dependent on the concentration of ethanol.

DISCUSSION

The results of these studies support the hypothesis that β-endorphin modulates the reinforcing effects of ethanol. Mice that express low levels of β-endorphin (50 percent of normal) drink more ethanol than wild-type mice. One possible explanation for this is that those mice with deficient basal levels of β-endorphin find the ethanol-induced release particularly reinforcing. Because ethanol cannot produce such a surge in genetically null mice, drinking might be particularly dependent on preabsorptive factors, and therefore more variable as a function of concentration, as seen in the homozygous knockouts

The enhanced administration of ethanol in mice with lower β-endorphin levels is concordant with previous findings from the clinical literature, as well as current thought underlying the use of antiopioid pharmacotherapies for alcoholism treatment (Herz 1997). The relationship between ethanol's effects and endogenous opioid peptides can be uniquely studied using mutant mice engineered for different levels of β-endorphin, and future directions are likely to include further neuropharmacological and behavioral analyses of the differential sensitivity between these strains to ethanol. It is hoped that these studies will contribute to our understanding of the relationship between opioids and ethanol and thereby expedite the successful treatment and prevention of alcoholism.

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